EXHIBIT B

Luminescent Labels—More than Just an Alternative to Radioisotopes?

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Chemical, chromatographic, or spectrometric methods are generally unsuitable for the detection of molecules in the nano- and subnanogram region because of their low sensitivity. The radioimmunoassay (RIA) developed by Yalow and Berson in 1959 combined the high sensitivity of radioactively labeled substances with the high specificity of immunological reactions for the first time. In this way it was possible to detect quantitatively the tiniest traces of substances in the presence of an excess of other, in some cases, similar foreign substances without prior enrichment. Immunoassays have certainly developed to become the most valuable analytical tool of in vitro diagnostics and are today routinely employed for the detection of endogenous and exogenous substances (e.g. hormones, tumor-associated proteins, bacteria, viruses, toxins, drugs, etc). The many disadvantages of radioactivity such as the required handling licenses, disposal costs, precautions necessary to prevent risks to health, short shelf-life, and limited sensitivity soon led to the search for other nonradioactive labeling methods. Encouraged by the development of light measuring techniques and the commercial availability of highly sensitive apparatus, radioactive isotopes as labels are today being replaced increasingly by enzymes, fluorophores, or luminophores. Some of the new luminescent labels have, however, not only facilitated replacement of radioisotopes, but also a breakthrough into what has until now been unattainable levels of sensitivity. The following article reviews the methods of luminescent labeling and their applications mainly in the area of immunoassays.

1. Introduction

The detection of substances with reagents which bind to the compound to be determined (analyte) is essentially dependent on three conditions if lower detection limits in the pico- to femtomolar region are to be attained and structurally similar substances are not to be measured in addition. First, the detection reagent must have a high affinity for the analyte so that even an analyte present in trace amounts is determined. Second, the binding of the detection reagent to the analyte should be highly specific; this ensures that substances similar to the analyte do not give rise to a deceptively higher concentration of the analyte, or make a time-consuming and labor-intensive prepurification necessary. Third, the reaction product from the analyte and binding reagent must be sensitive to detection, that is, emit a signal which can be quantified exactly by suitable analytical instruments.

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The demands for affinity and specificity are ideally fulfilled by antibodies. This involves endogenous glycoproteins, which in organisms of higher life forms, play a decisive role within the immune system by eliminating harmful substances (bacteria, viruses, toxins etc.). In general, affinity constants of antibodies lie between 10^{10} and $10^{12}\,L\,mol^{-1}$. They are capable of recognizing the smallest structural differences at the molecular level, because only then can they distinguish reliably between exogenous and endogenous substances; confusion between the two would have fatal consequences for the host organism.

The first methods used to label detection reagents and thus make them exactly quantifiable employed radioactive isotopes, of which the ^{125}I isotope, in particular, is still in use today. The advantages of this γ emitter are its small size (minimization of steric interference), its "hard" signal, which as a result is less prone to interference, and its lower detection limit of approximately 10 amol (1 amol $=10^{-18}$ mol).

The combination of antibody/radioactive labeling led to the introduction of radioimmunoassays at the end of the 1950s.[1] These have developed into the most important tool of in vitro diagnostics in medicine.[2] All conceivable endogenous and exogenous substances in the body fluids (e.g. blood or serum) taken from a patient are routinely determined quantitatively by radioimmunoassays. Of considerable importance for routine applicability is that despite the complex composition of the serum medium under investigation, in general, further purifica-

tion steps are not necessary because of the high specificity of the detection reagent "antibody".

Besides the advantages of radioactive labeling there are also, however, a number of disadvantages: The handling of radioactive materials is regulated (official license) and is, thus, limited. The half-life of 60 days for ^{125}I isotope is too short to guarantee a longer shelf-life for labeled reagents. Limited signal emission during measurement due to the natural half-life prevents the high detection sensitivity required for some applications.

In order to overcome these disadvantages the search for nonradioactive labeling methods (nonisotopic methods) in immunodiagnostics had already begun long ago.[3] At the beginning of the 1970s the use of enzymes as labels was described,[4] and in the meantime a large number of stable enzyme labels have become available. In combination with chromogenic or luminogenic substrates, detection of the signal is attributed to the measurement of light as absorption or emission. Emitted light instead of radioactive radiation is also employed in luminogenic direct labeling for the quantification of the analyte concentration. Since the number of photons from samples of luminescent-labeled molecules can be higher than the number of radiation quanta emitted from radioisotopes,[5] initially fluorescence detection seemed to have a good chance of a wide application in nonradioactive labeling;[5,6] for example, detection of a single fluorescent-labeled protein molecule was successful.[7,8] However, because of certain disadvantages associated with the first fluorescent label a signifcant replacement of the radioactive label was not forthcoming. This was only achieved by further development of luminescent labels and luminogenic enzyme substrates.[9-15] Luminescent labels not only dispense with having to handle radioactivity but they also allow more precise diagnostic results on account of enhanced sensitivity, and open up new areas of application.

In 1985 the radioimmunoassay (RIA) dominated the German immunoassay market (83% share) with the greatest turnover in the indication fields endocrinology (thyroid gland, fertility) and tumor diagnosis (Fig. 1). The tendency towards the use of methods with nonisotopically labeled compounds is shown in the statistics for 1991; the RIA share of the market has sunk considerably to 55%.[16] The market share of nonradioactive methods comprises luminescence techniques (e.g. fluorescence immunoassay (FIA), chemiluminescence immunoassay (CIA)), enzyme immunoassay (EIA), and other methods such as nephelometry. A further increase in the market share for methods that work without isotopically labeled compounds is to be expected in the future.

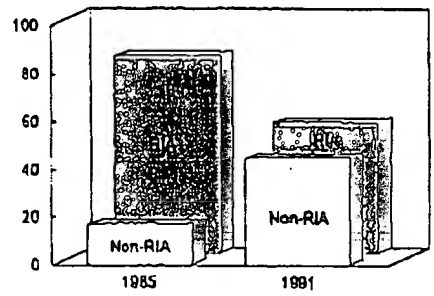

Fig. 1. Market share of radioactive (RIA) and nonradioactive immunoassays (non-RIA).

This review article deals in the broadest sense with luminescent labels and their application. After a definition of the term "label" in Section 2 luminescent processes are presented in a simplified energy-level diagram in Section 3. Section 4 describes the enzyme labels in combination with chromogenic and luminogenic substrates. In Section 5 labels for luminescent direct labeling with special consideration of the underlying mechanisms of chemiluminescent labeling are discussed. Section 6 presents several examples of applications in medicine; further important areas of application are in environmental and food analysis.[17a-b,18a-p] In addition to the literature already mentioned, the theme of luminescent labels, nonradioactive immunoassays, and gene probes is referred to in several recent books[17] and review articles.[18]

Andreas Mayer, born 1960 in Aschaffenburg, studied chemistry at the Universität Würzburg and received his doctorate under the supervision of H. Quast for work on dicyanosemibullvalenes. In 1989 he entered the main laboratory of the Hoechst AG and strengthened the team working on the development of chemiluminescent labels for diagnostic applications. The main emphasis of his work so far has been the synthesis of functional dyes for applications in the area of diagnostics and information technology.

Stephan Neuenhofer, born 1955 in Mayen/Eifel, studied chemistry and pharmacy in Bonn. After completing his diploma in chemistry (1982) he moved into biochemistry and received his doctorate in 1985 with K. Sandhoff on the topic of gangliosides (Lysogangliosides—Synthesis, Detection in Pathological Brain Tissue and Applications in Biochemical Studies). For his dissertation he received the Edmund-Ter-Meer-Preis. In 1987 he received his approbation as a pharmacist. In August 1987 he began working in the research and development department at the Hoechst AG. His area of interests comprises luminogenic labeling substances, immunoassays, and diagnostic systems as well as their transfer to production. He moved to Behringwerke AG in October 1993 where he has continued with these avenues of research.

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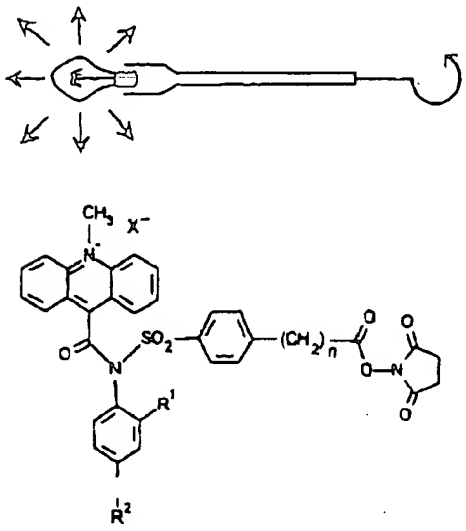
2. The Label

2.1. Definition and General Structure

A label is a molecule capable of emitting a signal, which is used for labeling proteins and other molecules. It contains, apart from the signal-generating group (fluorescer; more specifically, label), another reactive group (anchor group) which facilitates the covalent bonding to the molecule to be labeled. Between these two groups there is usually a spacer which is supposed to prevent, or at least make difficult, undesirable steric interactions between the signal-generating group of the molecule and the labeled substance. In this way any influences on the immunological reaction ought to be excluded. The schematic structure of a label as well as the chemical formula of a chemiluminescent label from the



Scheme 1. Schematic representation of a luminescent label (top) with the three components: signal-generating unit (fluorescer), spacer, and anchor group as well as a concrete example from the *N*-methylacridinium-9-(*N*-sulfonyl)carboxamide class of compounds (bottom).

class of *N*-methylacridinium-9-(*N*-sulfonyl)carboxamides is shown in Scheme 1. A substance labeled in this way is designated as a tracer.

2.2. Luminescent Labels

2.2.1. Definitions of Terms

The term luminescence[19] serves as the generic term for most light emission processes such as fluorescence, phosphorescence, chemiluminescence, electroluminescence etc. Exceptions are, for example, glow emission and coherent scattering processes. In practice, three categories are often used, namely luminescence, fluorescence, and phosphorescence. Luminescence serves as the generic term for chemi- and bioluminescence.

2.2.2. Requirements for the Suitability as a Luminescent Label

The suitability of a compound as a luminescent label has certain conditions which must be fulfilled:[20]

- Coupling to the compound to be analyzed (analyte) must be simple and quite gentle. A large palette of reactive groups is available for this purpose.
- The luminescent properties of the label should not change significantly after the coupling.
- The properties of the labeled substance must not be altered significantly by the labeling. The whole spectrum of characteristics must be taken into consideration, for example physico-chemical properties such as solubility and immunological properties. For the duration of the immunoassay, the immunological reactivity, in particular, must remain sufficiently high.

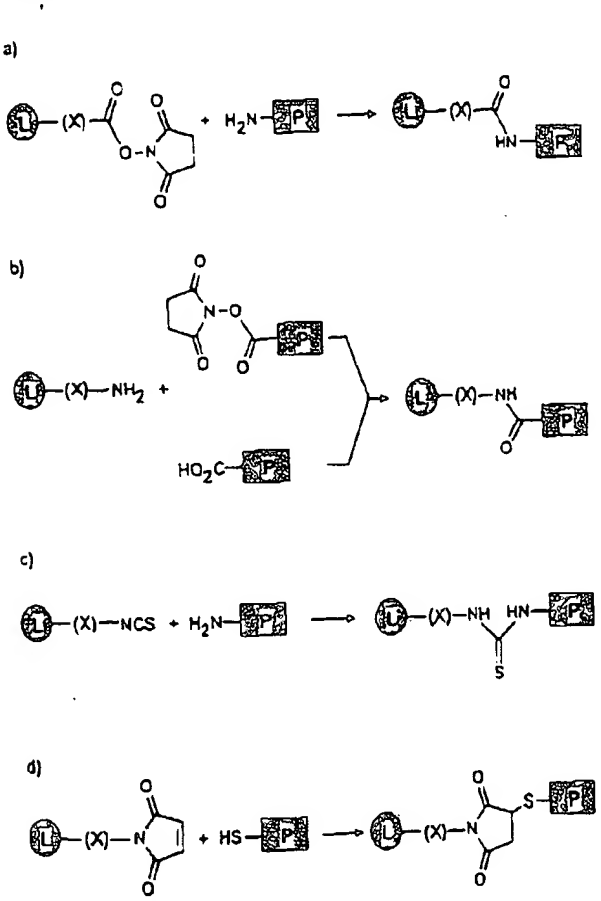
Of course, these general requirements, with the exception of the second point, are also applicable to other labels. The extent to which these points are fulfilled greatly depends on the details of each system and differs from case to case. For instance, small molecules (molecular weight <2 kD) are altered more significantly than large proteins when labeled with a marker of similar size. Proteins, however, are often more sensitive under the labeling conditions; for example, syntheses cannot be carried out in organic solvents. Nevertheless, for most, the conditions to yield suitable conjugates are established by chemical modification of the signal-generating group and/or of the spacer and by choice of the optimal reactive group.

2.2.3. The Signal-Generating Group

Fundamentally all luminescent compounds can be considered to be signal-generating groups if they exhibit a sufficient quantum yield in aqueous solutions, are stable enough under the conditions employed, and can be functionalized synthetically in such a way that a reactive group can be bound and the properties can be modified, for example, to increase the solubility in water or to change emission characteristics such as wavelength and decay time. Variability is a prerequisite for the broadest possible application of the label. The most important fluorescent and chemiluminescent labels are considered in more detail in Section 5.[7a, 21]

2.2.4. The Reactive Group

As mentioned previously, the reactive or anchor group is used to bind the label to the substance to be labeled. Since coupling to biological material such as proteins, antibodies, hormones etc. is frequently necessary, the formation of an acid amide bond between activated carboxyl groups and amino groups is quite common. Many processes for this are known in peptide chemistry which proceed in aqueous solution under mild conditions.[22, 23] However, only a few reactions have achieved practical significance for labeling processes. Some of the most important coupling reactions are summarized in Scheme 2. Many luminescent labels have an *N*-hydroxysuccinimide (NHS) ester as the reactive group (Scheme 2a).[21, 25] This reactive group has several advantages:[22, 24] it can be readily synthesized from carboxylic acid derivatives;[20] corresponding labels can be purified to a high degree, for example, by HPLC; thus, labeling can be carried out

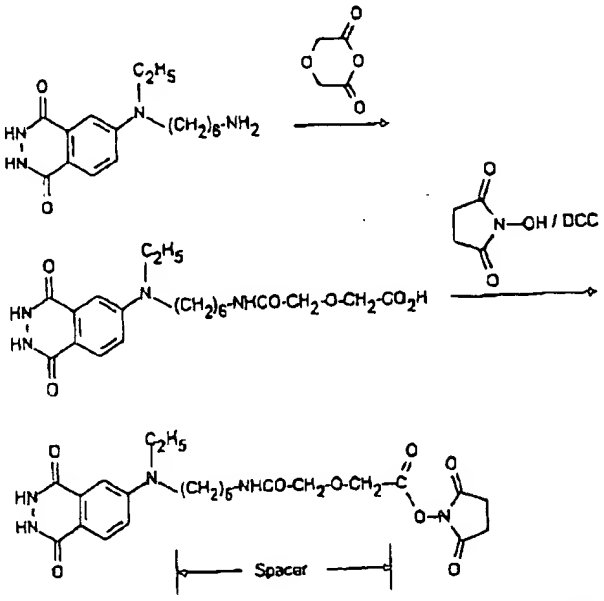


Scheme 2. Important labeling processes: L = label, P = protein, X = spacer.

in a defined and reproducible way. With the exclusion of moisture it is possible to store the label over longer periods of time without the reversal of the coupling activity.[24, 27, 28] The coupling reaction of the succinimide with amino groups proceeds under mild conditions (room temperature) in aqueous solutions; in contrast, alcohols do not react with NHS esters under these conditions.[27] As a variation of this reaction the label can also contain a primary amino functionality as the reactive group for coupling with an NHS ester functionality on the protein (Scheme 2b). The conversion can also be achieved with free carboxyl groups, for example, of proteins, following carbodiimide or "mixed anhydride" methods.[22, 23, 25] Recently acridinium ester labels with imido ester reactive groups were described.[29] Particularly in the case of fluorescent labels, in addition to the the methods already mentioned the isothiocyanate group is often used for coupling with amino groups of proteins to form thiourea derivatives (Scheme 2c).[30] The method shown in Scheme 2d for the coupling by thiol addition to maleic imido groups is well known in peptide chemistry. This reaction is also employed in luminescent labeling[24] and plays a particularly important role in the coupling of enzyme labels to proteins.[31]

2.2.5. The Spacer

Usually simple, short alkyl chains or groups which contain aromatic and aliphatic groups (cf. Scheme 1) are employed as

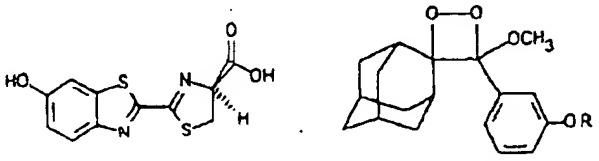


Scheme 3. Synthesis method for a spacer with an NHS reactive group as illustrated by an example of an isoluminol label; DCC = dicyclohexylcarbodiimide.

spacer. An example of the conversion of an amino group of an isoluminol label to give an NHS ester is shown in Scheme 3.[32] By using a spacer of the type shown, which contains several acid amide groups and ether bridges as hydrophilic units, the solubility of immunoconjugates in water can be improved.[33]

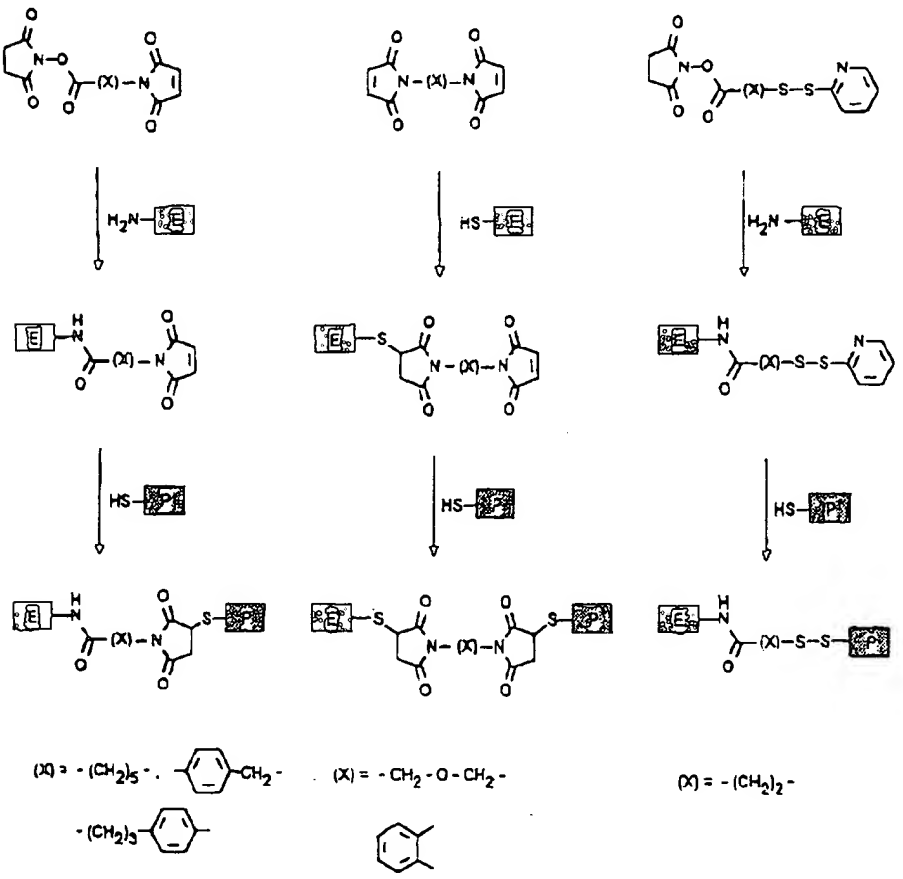
2.3. Enzyme Labels

As a result of their practical significance chromogens and luminogens are also dealt with in this article; these are employed as substrates for the enzyme label (Section 4). The reaction of luciferin derivatives (naturally occurring bioluminescent compounds) with their respective luciferase (enzyme which catalyzes the bioluminescent reaction) leads to luminescent reactions with the highest known quantum yields. The leader in this field is the luciferin (Scheme 4)/luciferase system of the North American



Scheme 4. Firefly luciferin (left) and a stable dioxetane derivative (right).

firefly (*Photinus Pyralis*) with quantum yields around 0.9 Einstein mol^{-1}.[13, 15] Since the end of the 1980s important biogenic luciferases have been genetically engineered and are thus now considered for routine applications.[15] High quantum yields are also obtained by enzyme labels with stable dioxetanes (Scheme 4) as luminogenic substrates. The enzymes often employed are alkaline phosphatase and β-galactosidase (the residue R is phosphate and β-galactose, respectively). Such enzymatic systems are dealt with in more detail in Section 4.



Scheme 5. Bifunctional coupling reagents for labeling of enzymes: E = enzyme, P = protein (e.g. antibody), X = spacer.

Since, in general, enzyme labels are not obtainable as stable universally applicable labels, several methods for enzyme labeling should be addressed. The coupling of enzymes to antibodies or fragments of antibodies is often achieved by bifunctional coupling reagents. Some examples are shown in Scheme 5. The reactions are analogous to the methods already mentioned in Section 2.2.4, and details can be obtained from the references cited in the literature.[24, 31] Coupling methods that exploit the strong noncovalent bonding of the biotin/(strept-)avidin system can only be referred to here.[34]

3. Electromagnetic Radiation as the Measured Signal

3.1. Comparison of Light and Radioactive Radiation

A considerable disadvantage in the use of radioactive isotopes is the necessity to undertake extensive safety precautions against high-energy β- or γ radiation (up to 10^{19} kJ mol^{-1}). Since luminescent labels emit light which is not dangerous—mostly in the visible region of the electromagnetic spectrum ($E \approx 200$ kJ mol^{-1})—safety measures for the protection from high-energy radiation are no longer required. Furthermore, the specific activity that can be achieved with radioisotopes has an upper limit set by the radiolytic decomposition of the labeled material. Labeling with the isotope ^{125}I is usually limited to one atom per molecule.[21, 33] In addition, the half-life of the radioisotope, which, for example, is only 59.7 days for the frequently used ^{125}I, limits the storability of the labeled material and detection limits. Moreover, radioisotopes emit radiation continuously even when a signal is not necessary for measurement. For the actual measurement, which normally lasts about a minute, only a tiny fraction of the available signal, can be used. An advantage, however, is that a repeat measurement is possible at any time. Luminescent labels, which have a considerably longer lifetime, emit all the available light within a very short space of time once the light reaction has been triggered. In addition, the activity of the tracer can be enhanced further mainly by multiple labelings; in this way the sensitivity of detection is increased. Repeat measurements on the same sample are, however, not possible, at least in the application of chemiluminescent labels with rapid light emission.

3.2. Photophysical Processes

In the simplified energy-level diagram in Figure 2 the most important photophysical processes are summarized with their typical lifetimes τ [s].[136] The radiative transitions shown can be used for the production of detection signals. Since radiationless deactivation leads to less efficiency, especially in long-lived phosphorescence processes in solution, phosphorescence detection plays a minor role for luminescent labels. Finally, the quantification in enzyme systems with chromogenic substrates (cf. Sec-

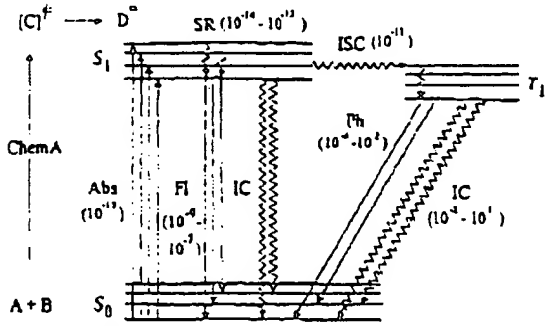


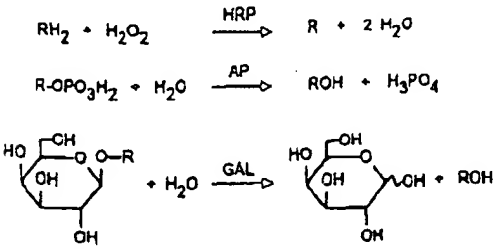
Fig. 2. Simplified energy-level diagram with some photophysical processes. Lifetime τ (s) is given in parentheses; Abs = absorption, Fl = fluorescence, Ph = phosphorescence, SR = vibrational relaxation, ChemA = chemical excitation, IC = internal conversion, ISC = intersystem crossing; straight lines represent radiative processes and wavy lines radiationless processes.

tion 4) is ascribable to absorption measurements. Photoexcitation and evaluation of the fluorescence provide the basis for fluorescent labeling (cf. Section 5.1). The production of excited singlet states (S_1) by chemical reactions is necessary for chemiluminescence detection. The difference in energy between the S_1 and S_0 states for emissions in the visible region lies between 167 (red light) and 293 kJ mol^{-1} (violet light).[11, 37] For the effective use of the principles mentioned, in each case, sufficient quantum yields are also a prerequisite. Further details are given for each individual luminophore (see Section 5).

4. Enzyme Labels

The use of enzymes as labels presented the first alternative to radioactive labeling.[38] The basic idea is very promising, because no signal-generating compounds are used, but molecules (enzymes) which produce a lot of signal-generating species. In this way an effective signal amplification mechanism is built in right from the outset.

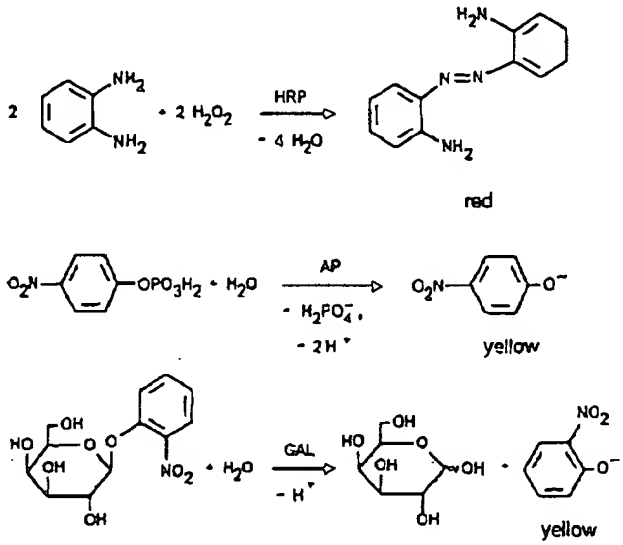
The three most important enzymes that are used as labels are horseradish peroxidase (HRP), alkaline phosphotase (AP), and β-D-galactosidase (GAL). The reactions catalyzed by these enzymes are summarized in Scheme 6. HRP is the smallest enzyme



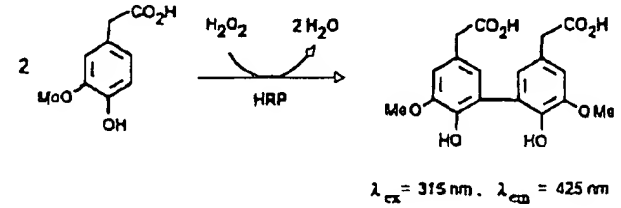
Scheme 6. General representation of the reactions catalyzed by the three most important enzyme labels HRP, AP, and GAL.

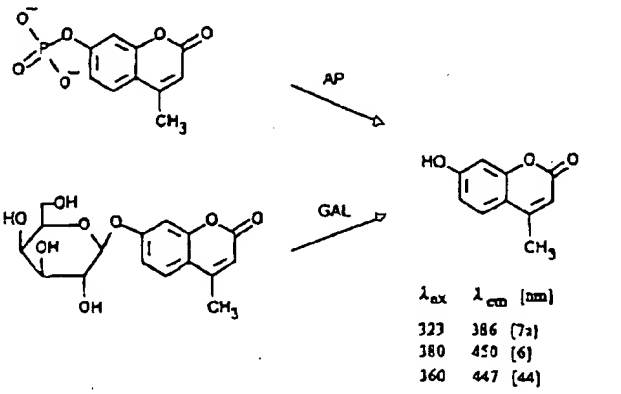
of the three with a molecular weight of approximately 40 kD (AP approx. 100 kD, GAL approx. 500 kD) and as a result presents the fewest steric problems. HRP is, however, sensitive towards antimicrobial agents (azide, Thiomersal) frequently employed. AP has the highest catalytic activity; however, it is inhibited by phosphate (product inhibition) and ethylenediaminetetraacetic acid (edta; chelates Zn^{2+} and Mg^{2+} ions which are necessary for enzyme activity).

The oldest enzyme substrates to be employed in analytical methods are the chromogens. These are colorless and are only transformed into colored products by an enzymatic reaction. These products can be quantified photometrically; Scheme 7 shows examples.



Scheme 7. Examples of chromogenic substrates. Apart from o-phenylenediamine, 3,3',5,5'-tetramethylbenzidine (TMB) and 2,2'-azinobis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) are often employed. Instead of 4-nitrophenylphosphate, 1-naphthylphosphate and 5-bromo-4-chloro-3-indolylphosphate are often employed. In addition to 2- or 4-nitrophenyl-β-D-galactopyranoside, chlorophenol red-β-galactopyranoside and 5-bromo-4-chloro-3-indolyl-β-galactopyranoside are often employed.

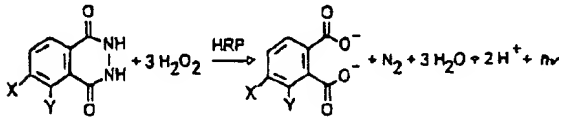




Scheme 8. Examples of fluorogenic substrates. λ_{ex} = excitation wavelength, λ_{em} = emission wavelength.

The most important fluorogenic substrates of the peroxidases contain the same structural element[39, 40, 43] *p*-HO–C_6H_4–C–, whereas in the case of AP and GAL,[41–43] 4-methylumbelliferyl compounds are preferred (Scheme 8).

The chemiluminescent arylhydrazides luminol and isoluminol[45] (Scheme 9) are known substrates for HRP. The quantum

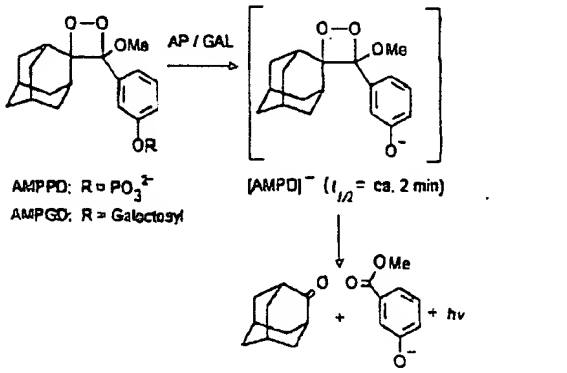


Luminol: X = H, Y = NH_2
Isoluminol: X = NH_2, Y = H

Scheme 9. HRP-catalyzed oxidation of luminol and isoluminol.

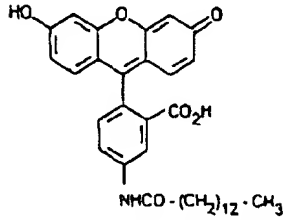
yields of the oxidation of these arylhydrazides, that is, the percentage of the molecules of the starting material which at the end of the reaction afford a photon-emitting final product, is approximately 1%. The light intensity can be drastically increased (up to a factor of 1000!) by so-called enhancers such as 6-hydroxybenzothiazole derivatives or *para*-substituted phenols in comparison to a non-enhanced reaction.[46] The underlying mechanism of this enhanced chemiluminescence is not completely understood. The most probable explanation is that one or more of the oxidation steps to generate luminol radicals during the complex reaction pathway of the enzymatic oxidation is accelerated.[46b]

Dioxetane derivatives are the most important chemiluminogenic substrates for AP and GAL[47] (Scheme 10). Also in the



AMPPD: R = PO_3^{2-}
AMPGD: R = Galactosyl
[AMPD]$^-$ ($t_{1/2}$ = ca. 2 min)

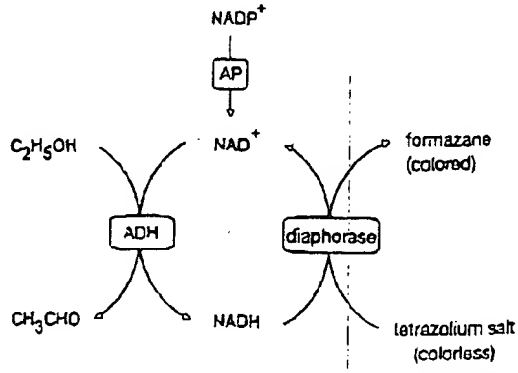
Scheme 10. Adamantylmethoxy(phosphoryloxyphenyl)dioxetane (AMPPD) and adamantylmethoxy(galactopyranosyloxyphenyl)dioxetane (AMPGD) as enzyme substrates. This reaction is catalyzed by AP and GAL.



Scheme 11. Example [5-(*N*-tetradecanoylamino fluorescein)] of a fluorescent tenside.

reactions catalyzed by AP and GAL, the chemiluminescence can be enhanced: for this purpose fluorescent tensides are particularly effective (Scheme 11). Together with normal tenside molecules (e.g. cetyltrimethylammonium bromide) they form micelles into which the substrate diffuses immediately after enzymatic cleavage of the phosphate or galactoside residue. Decomposition of the dioxetane derivative in the micelle leads to an effective transfer of energy to the fluorophore groups of the tenside molecules. This results in a considerable increase in the quantum yield[47] (Φ_{CL} = approx. 0.005 Einstein per mol; for comparison: Φ_{CL} in tenside-free aqueous buffer solution is ca. 10^{-5} Einstein per mol). According to the literature, as few as 600 different enzyme molecules have so far been detected by employing dioxetane derivatives.[47e]

In the detection systems described previously the signal-generating group forms immediately in the reaction catalyzed by the enzyme label. There is also, however, a series of detection systems in which the signal-generating group is formed only in a subsequent reaction. In analogy to enzyme-catalyzed reactions, considerable increases in the sensitivity can often be achieved by such coupling reactions. In the detection system of Self,[48a] the enzyme label AP catalyzes the formation of NAD^+ from $NADP^+$. The NAD^+ formed catalyzes a specific redox cycle from which a colored substance is produced (Scheme 12).



Scheme 12. Example of a signal amplification: The NAD^+ formed from AP acts as a catalyst in a subsequent redox cycle. ADH = alcohol dehydrogenase.

The lower detection limit for the enzyme label AP with this method was given as 0.01 amol,[48b] and in a more recent publication[48c] even 0.6 zmol (1 zeptomol = 10^{-21} mol); values which would have been inconceivable for direct formation of a dye.

Another method for the highly sensitive detection of AP was described by Christopoulos and Diamandis.[49] AP catalyzes formation of 5-fluorosalicylic acid from 5-fluorosalicylic phosphate. In a subsequent reaction 5-fluorosalicylic acid forms a strongly fluorescing ternary complex with Tb^{3+} and edta the concentration of which can be quantified by time-resolved fluorescence measurements (cf. Section 5.1.4). The lower detection limit was quoted as being 0.6 amol AP per 50 µL sample volume. In the detection system of A. Baret et al.[50] xanthine oxidase is used as the enzyme label. In the presence of oxygen, it oxidizes hypoxanthine to xanthine and uric acid with formation of superoxide radical anions ($O_2^{\cdot-}$). Additional reactive oxygen species (H_2O_2, $^1O_2$, $OH^{\cdot}$) that form in subsequent reactions are suitable for the chemiluminescent oxidation of luminol.

5. Luminescent Labels

5.1. Fluorescent Labeling

Fluorescent labels have been used for a wide range of applications in biology, biomedicine, and analytic methods for a long time.[30] Applications worthy of mention are fluorescence detection with HPLC after precolumn or postcolumn derivatization,[51] flow cytometry,[52] fluorescence microscopy,[53] DNA analysis,[54] and the use as labels in immunoassays, which will be covered in more detail here (see Section 5.1.1). Labeling is usually achieved by the formation of a covalent bond between label—as described, in general, in Section 2—and target substance. Fluorescent dyes without a reactive group can be employed for some purposes. They are only bonded associatively and as a result can accumulate, for example, in cells. A review of fluorochromes that are applied in medicine and biology as well as their spectral data can be found in reference [36].

5.1.1. Special Requirements of Fluorescent Labels for Immunoassays

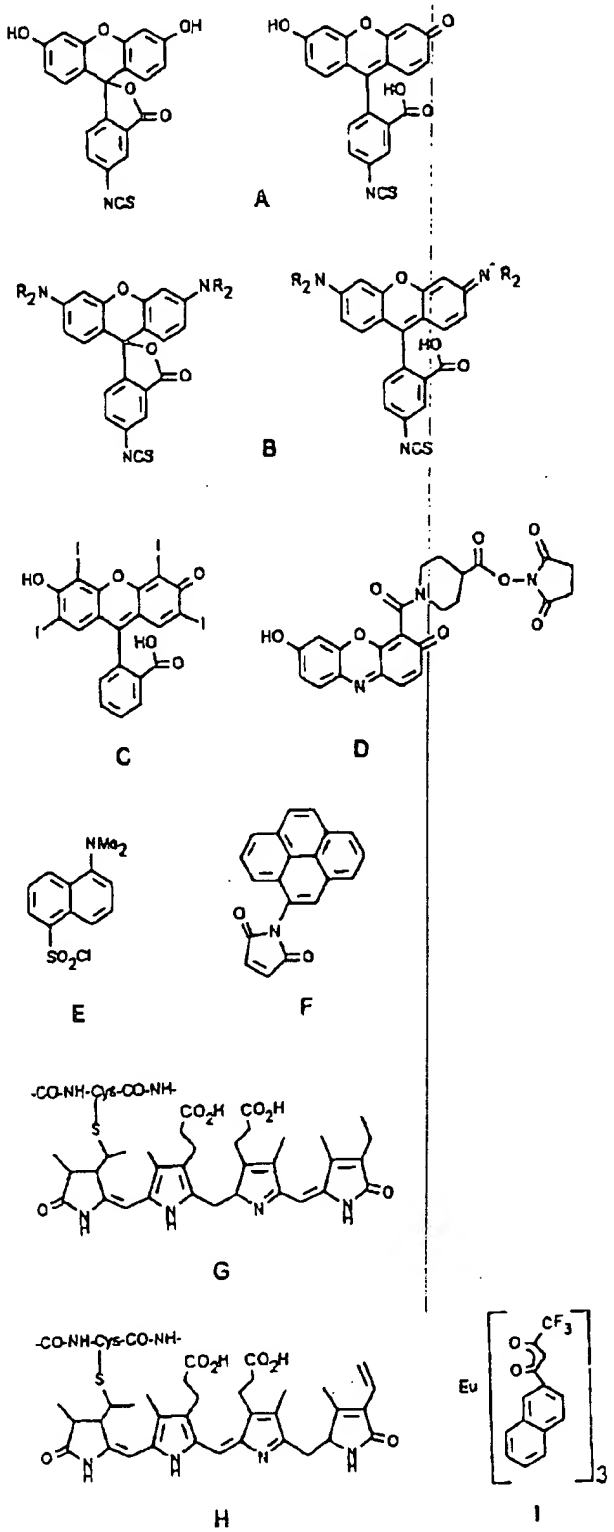
Besides the previously mentioned general requirements for luminescent labels, fluorescent labels for the use in immunoassays should fulfill a few additional conditions which can be derived directly from measured data. In principle, fluorescence measurements of the highest sensitivity are indeed possible,[6, 7, 55] but, in practice, the sensitivity of fluorescent immunoassays (FIAs) is, however, drastically limited by background fluorescence, light scattering and quenching effects. The intrinsic fluorescence of serum components is mainly responsible for the background signal[6, 7] which covers a broad wavelength region. Serum proteins are excited, for example, at 280 nm and emit at 320–350 nm. Other components such as NADH and bilirubin are excited between 330 and 360 nm and 450 and 460 nm, respectively, and fluoresce in the range 430–470 nm and 515 nm, respectively.[7c] The detection limit for immunoconjugates of a fluorescent label with bovine serum albumin or immunoglobulin G (IgG) is on average 10 to 50 times worse in serum than in buffer solution.[6a] Many solid phase materials, for example polystyrene, likewise yield a blank reading. Light scattering is a problem, particularly, in solutions which contain proteins or colloidally dispersed substances. In addition to Rayleigh and Tyndall scattering at the same frequency as the excitation beam, Raman scattering also occurs with a frequency usually shifted by approximately 50 nm. Fluorescence quenching can often result from the smallest changes in the environment of the fluorophore (pH, polarity, oxidation level, proximity of heavy atoms or other absorbing groups). If, for example, a protein is multiply labeled, two fluorophores can become so close that self-quenching of the signal takes place if the absorption and emission spectra overlap.

In order to minimize the influences mentioned the following properties of fluorescent labels are desirable:[30] a) longest possible wavelength emission (500–700 nm), b) large Stoke's shift of > 50 nm, c) long fluorescence lifetime of $\tau > 20$ ns.

A sufficiently long lifetime is particularly significant in applying the principle of fluorescence polarization transfer (cf. Section 5.1.3). Fluorescence lifetimes $\tau > 100$ ns facilitate a significant improvement of the signal-to-noise ratio and thus of the sensitivity, since measurement can only take place after decay of background fluorescence and light scattering. This principle is applied in time-resolved fluorescence measurements which are explained in more detail in Section 5.1.4.

5.1.2. Labels for Direct Fluorescent Labeling

The first compound used for fluorescent labeling of biological material by Coons et al. in 1941 was anthracene isocyanate for the labeling of bacterial proteins.[57] The same group introduced fluoroscein isothiocyanate (FITC, A, Scheme 13) as a more ef-



Scheme 13. Selected fluorescent labels A–I. See Table 1.

fective label soon afterwards.[58] Although judging from its spectral data this xanthene dye (cf. Table 1) does not completely fulfil the above-mentioned requirements for fluorescent labels,

Table 1. Spectral data of selected fluorescent labels A–I.

	$\lambda_{excitation}$ [nm]	λ_{em} [nm]	ε [L mol^{-1} cm^{-1}]	τ [ns]	Φ
fluorescein isothiocyanate A	492	520	7×10^4	4.5	0.85
rhodamine B isothiocyanate B (R = Et)	550	585	[a]	3.0	0.7
TM-rhodamine isothiocyanate B (R = Me)	550	580	5×10^4	2.0	
erythrosine C	530	558 fl [b] 690 ph [b]	1×10^5	2.7×10^5	0.002
resorufin derivatives D	575	590			
dansylchloride E	340	480–520	3.4×10^3	14.0	0.3
pyrene maleimide F	340	375, 392	3.8×10^4	100	
phycocyanobilin, protein bound G	614	643	5.8×10^5	2.2	0.52 [c]
phycoerythrobilin, protein bound H	546	576	2.4×10^5	3.2	0.59 [c]
europium-tris(2-naphthoyltrifluoroacetone) I	340	590, 613	3.6×10^4	7×10^5	0.8

[a] Unclear literature values: 12300 [6a,b]; 10300 [7a]. b) fl = fluorescence; ph = phosphorescence. c) Most phycobiliproteins afford Φ values up to 0.98.

FITC has become the fluorochrome of choice in most applications.[9, 10] Despite the large number of fluorescent labels which have been developed since, FITC still remains the most commonly used in fluorescence immunoassays, possibly due to a high quantum yield and stability. Similar properties are exhibited by rhodamines B which belong to the same class of dyes (Scheme 13). Both dyes can exist in the two isomeric forms shown, a spirolactone and quinoid structure.

Efforts to obtain fluorescent labels that can be excited in the longwave region and also emit revealed, for example, that derivatives of the phenoxazin dye resorufin were successful to a certain extent. In the synthesis of fluorescent labels, suitably functionalized resorufins are obtained, for example, from nitrorcesorcin and 2,6-dihydroxybenzoic acid after reduction of the initially formed resazurine (resorufin-*N*-oxide). Apart from the derivative D shown in Scheme 13 which has a succinimidoyl ester as the reactive group, labels based on resorufin with other reactive groups are also known. Compared to fluorescein, resorufin is less affected by the background fluorescence of serum.[59] A longwave shift of excitation and emission wavelengths is also possible with phycobiliproteins,[60] which are obtained from different kinds of red and green algae. The structures of the two prosthetic groups are given in Scheme 13 (G, H). The compounds exhibit very high molar extinction coefficients and high quantum yields (> 0.8).[61] Not all phycobiliproteins couple with the protein at the A-ring. The substances, which have, in the meantime, become commercially available, were first employed in fluorescence microscopy and flow cytometry, thereafter as labels in immunoassays. When, for example, fluorescein was replaced by phycoerythrin in a sandwich immunoassay, a significant increase in sensitivity (factor 2–10) was achieved; however, this was below that expected from the spectral data.[61, 62] The size of the label and the difficulty in coupling are unfavorable. Since the phycobiliproteins show a broad excitation and emission band, a parallel determination of several parameters is conceivable by the use of different labels with non-overlapping emission bands. This was confirmed in preliminary experiments.

5.1.3. Fluorescence Polarization

The principle of fluorescence polarization,[63] known for a long time, was first employed in the antigen–antibody reaction in 1961.[64] Fluorescence polarization immunoassay is based on the following principle: if a fluorescing compound in solution is excited by polarized light, the observed emission is also polarized. The degree of this polarization depends upon the rotation relaxation time and, thus, on the size of the molecule. If a small (M: 1–10 kD) fluorescent-labeled fast-rotating molecule is bound to an antibody ($M \approx 160$ kD), the result is an increase in the rotating relaxation time of the slowly tumbling immunocomplex and thus also the polarization of the fluorescence. With this principle one can differentiate between unbound labeled antigen and immunocomplex. The method is, however, not suitable for large antigens, since the rotation hardly changes on formation of the immunocomplex. A more exact derivation of the measurement principles is given in reference [63]. The fluorophores mentioned already can be used as labels, although substances with longer fluorescence lifetimes would be more advantageous. In most cases fluorescein isothiocyanate A is employed. Accordingly, the sensitivity is limited by factors mentioned already.

Since no separation step is necessary with this principle (homogeneous immunoassay), determinations can be carried out relatively easily provided that sensitivity in the picomolar region is not required. Fluorescence polarization immunoassay is widely used particularly in the area of drug analysis.[59, 65] This method can also be employed in environmental analysis, for example, in the determination of polychlorinated biphenyls (PCBs). Fluorescein derivatives are used as labels.[65]

5.1.4. Time-Resolved Fluorescence

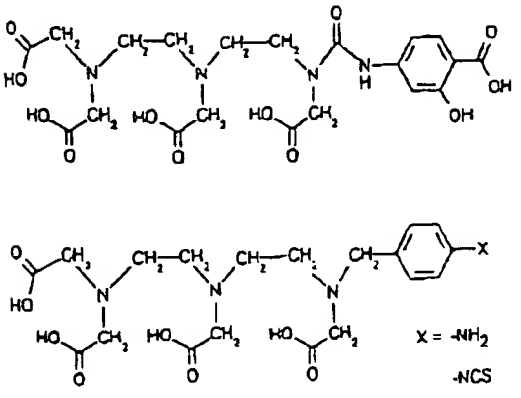
It has already been mentioned that the limiting background fluorescence and the scattering effects can be excluded by the use of labels with very long fluorescence lifetimes and by taking measurements only when the background signal is no longer present. Generally, the lifetime of the unspecific background signal is less than 10 ns. For an interference-free measurement of a specific signal, labels are required whose lifetimes are at least 10 times the decay time of the background.[66] Suitable organic fluorophores with lifetimes $\tau > 50$ ns are, however, very rare. Pyrene derivatives such as F in Scheme 13 exhibit lifetimes of approximately 100 ns, which, however, were shown to be insufficient.[66] The lifetimes of phoshorescence processes are considerably longer. The principle applicability of phosphorescent labels, such as erythrosin derivatives in oxygen-free solutions, could indeed be shown[67] but no progress with phosphores

cence was achieved due to its low quantum yields and high expenditure.

It was not until complexes with rare-earth metal ions, primarily europium(III), were employed as labels that prospects of attaining a drastic improvement in the sensitivity of fluorescence immunoassays by time-resolved measurements and developing more sensitive fluorescent alternatives to RIA were realized.[6, 66] The use of europium trisdiketonates was proposed by Wieder[76] in 1978 and developed further by other groups.[68]

The chelate complexes of europium(III), terbium(III), samarium(III), and dysprosium(III) are distinguished by unique fluorescent properties (cf. I in Scheme 13 and Table 1). Apart from extremely long lifetimes of about 1 μs to 1 ms, a very large Stoke's shift (>200 nm) and sharp emission lines impart the complexes with a high sensitivity (10^{-14} mol L^{-1}).[6, 66] The reason for the observed lifetimes lies in the excitation/emission mechanism. After excitation of the ligand to the S_1 state and intersystem crossing to an energetically suitable triplet state of the ligand, an effective energy transfer to the resonance state of the metal ion occurs, which then gives rise to a sharp emission characteristic of metal ions.[6, 66, 69]

The fluorescent properties of chelates of rare-earth metals alone still do not produce an efficient label for immunoassays. What is essential, also in aqueous buffer solution, is a stable binding to antigens and antibodies. Due to their high stability and solubility in water, polyaminopolycarboxylate chelates, chiefly derivatives of ethylenediaminetetraacetic acid (edta) or diethylenetriaminepentaacetic acid are used for most applications. The use of diazo- and isothiocyanatophenylethylenediaminetriacetate for coordination to europium(III) and terbium(III) has been described.[66, 70] Likewise, mixtures of ethylenediaminetriacetic acid, terbium(III), and 5-sulfosalicylic acid,[70, 71] mixtures of an edta derivative, europium(III), and a β-diketone[70, 72] as well as diethylenetriaminetetraacetic acid derivatives with different trivalent lanthanides[70, 66, 70, 73] were employed for the labeling (Scheme 14).



Scheme 14. Ligands for the coordination of lanthanide ions.

Since many of the very stable lanthanide chelate complexes do not fluoresce with these ligands, a dissociation step must be carried out prior to detection.[74] Furthermore, once the immune reaction is complete, a so-called enhancement solution is added after washing, which leads to the dissociation of the chelate complex and to the formation of fluorescent complexes [DELFIA system (*D*issociation *E*nhanced *L*anthanide *F*luorescence *I*mmuno-*A*ssay)]. The 1,3-diketones β-naphthoyltrifluoroacetone and pivaloyltrifluoroacetone are commonly employed.

The use of chelate complexes with trivalent lanthanide ions facilitated not only the development of highly sensitive immunoassays by time-resolved measurement, but also the simultaneous determination of several parameters, since Eu^{III}, Tb^{III}, Sm^{III}, and Dy^{III} complexes emit at considerably different wavelengths and have different fluorescence lifetimes. Several double determinations have been described;[73, 75] Eu^{III}/Tb^{III} chelates[76] or Eu^{III}/Sm^{III}[76] complexes were used as label pairs. In simultaneous determinations of two parameters from each, the dissociation/enhancement principle was employed. A simultaneous and highly sensitive determination of more than two lanthanide labels is, however, not possible with the simple enhancement solutions.

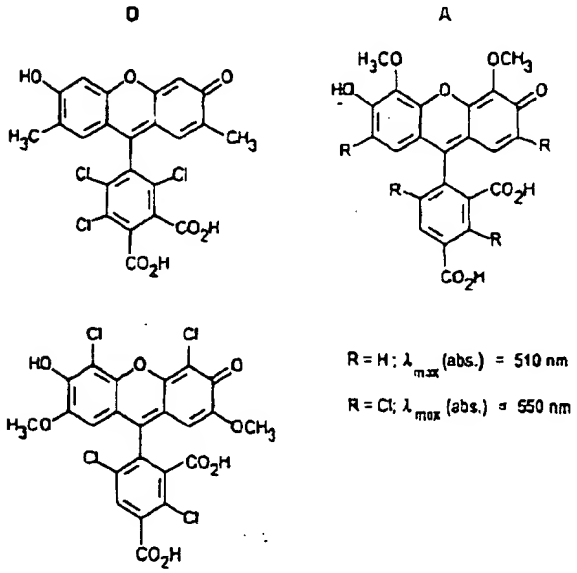
Simultaneous multianalyte determinations are gaining interest,[78] since with the four lanthanide ions mentioned and specially developed enhancement solutions, so-called co*fluorescence*-based enhancement solutions (CFES),[79] four parameters could be determined simultaneously by using time-resolved fluorescence measurements.[73] The enhancement solutions formed in this way consist of a dissociation element, pivaloyltrifluoroacetone and Y^{III}, as well as an element, 1,10-phenanthroline which enhances the fluorescence. Europium(III) and terbium(III) chelates with macrobicyclic ligands that contain α,α′-bipyridine or 1,10-phenanthroline units were already described earlier as efficient luminophores which act as molecular light transformers.[79c]

5.1.5. *Fluorescence Energy Transfer*

In the application of fluorescent labels for immunoassays, the principle of fluorescence-polarization, which facilitated development of homogeneous immunoassays, has already been mentioned. Another method, in which no separation of the unbound labeled molecules from the immunocomplexes is necessary, uses fluorescence energy transfers.[6b, 80] In this case an energy transfer from an electronically excited fluorophore (donor) to a neighboring acceptor dye molecule (quencher) occurs by dipole–dipole coupling. According to Förster[81] the efficiency of the energy transfer is indirectly proportional to the power 6 of the distance. With Förster's theory distance measurements in molecules, for example, can be obtained,[82] and for efficient energy transfer distances must not be greater than 10 nm. This condition is fulfilled in many antigen–antibody complexes. If, for example, the antigen is labeled with the donor and a specific antibody is labeled with the acceptor, quenching of fluorescence (of the donor) occurs in the immunocomplex. In a mixture of labeled and unlabeled antigens the fluorescence signal increases with the quantity of the unlabeled analyte to be determined.

The requirements for the fluorescent labels (donors) that should be employed in energy-transfer immunoassays are the same as those for fluorescent labels already mentioned. Furthermore, the choice of the donor–acceptor pair must be such that the emission spectrum of the donor and the absorption spectrum of the acceptor overlap well. In the beginning the use of the donor–acceptor pair fluorescein isothiocyanate/tetramethyl-

rhodamine isothiocyanate was described.[80b] Since interference from background signals such as background fluorescence from serum are particularly large in homogeneous immunoassay, standard labels such as fluorescein isothiocyanate, umbelliferones, or dansyl chloride certainly are of little significance. For the latter, in addition, the high sensitivity for background effects is disadvantageous. The same can be said for rhodamines such as tetramethylrhodamine. Due to higher absorption and emission wavenumbers, phycobiliproteins and lanthanide chelates are better suited as donors. With the latter, particularly in conjunction with time-resolved measurements, the development of more sensitive fluorescence energy-transfer immunoassays is possible. Also substituted fluoresceins with absorption and emission wavelengths greater than 500 nm (cf. Scheme 15) were used as donors.[80, 83]



Scheme 15. Fluorescein derivatives employed in fluorescent energy transfers; D = donor molecules, A = acceptor molecules.

The energy acceptors (quencher labels) should ideally fulfill the following conditions:[80a] a) high extinction coefficient of the emission wavelength of donor; b) no fluorescence during excitation in the absorption maxima of donor; c) good solubility in water in order to facilitate multiple labeling with the quencher (greater quenching effect); d) the smallest possible background interference in the absorption spectrum.

Since frequently used acceptors such as tetramethylrhodamines do not fulfill these requirements, new non-fluorescent fluorescein derivatives were described which form effective pairs with the donors in Scheme 15.[80, 84]

Fluorescent labeling and the principle of fluorescent energy transfers have recently also found application in the development of biosensors.[85] Detection can be based on the quenching of emission from the donor, new emission from the acceptor, or on the ratio of both emission wavelengths. One biosensor principle based on Langmuir–Blodgett films and fluorescence energy transfer with a cumarin derivative as donor and tetramethylrhodamine as acceptor was recently described.[86]

5.2. Chemiluminescent Labeling

A considerable difference between chemiluminescent detection systems and fluorescent labels is that former do not require the irradiation of the excitation light. With these chemiluminescent systems, in particular, in working with serum, the problems with high background signals, which are mainly responsible for the limited sensitivity of many methods with fluorescence detection, are prevented. However, in several chemiluminescent labels, complex systems comprising oxidation reagents, signal enhancer additives, and catalysts can likewise lead to an unacceptable high background signal, which, of course, has an adverse effect on sensitivity.[87] In the case of chemiluminescence detection in analysis systems, which above all are applied in medical diagnostics, compounds mainly from the following categories are employed:[9, 10–15, 17] luciferins in combination with the corresponding luciferases, cyclic arylhydrazides, acridinium derivatives, stable dioxetanes, and oxalic acid derivatives.

5.2.1. Bioluminescence

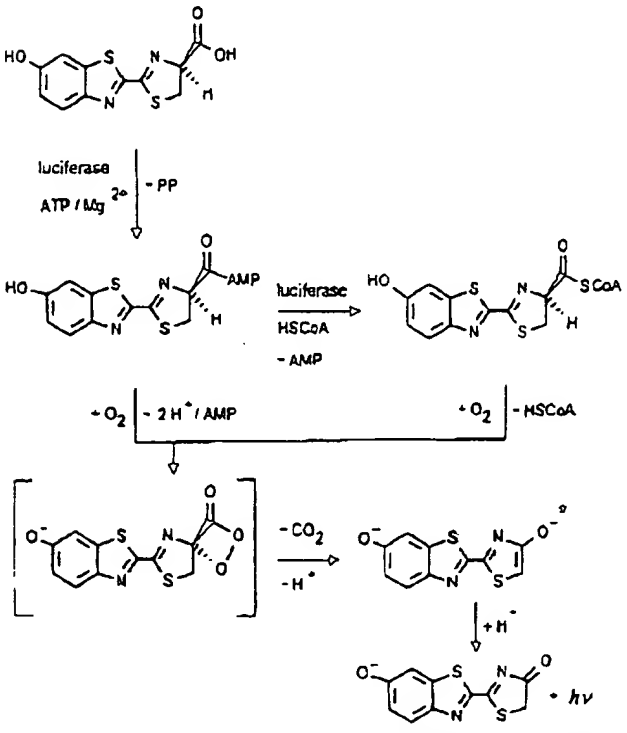
The luciferin/luciferase system of the North American firefly

One of the most well-known and most studied light systems in nature "operates" in the North American firefly (*Photinus Pyralus*). Although the mechanism of bioluminescence has been studied for more than 30 years, and the benzothiezole derivative luciferin became available synthetically and was structurally determined at the beginning of the 1960s, not all the details of the bioluminescence reaction have been elucidated. Since a more detailed description of this and other bioluminescent systems would go beyond the framework of this review article only the latest developments are described briefly.

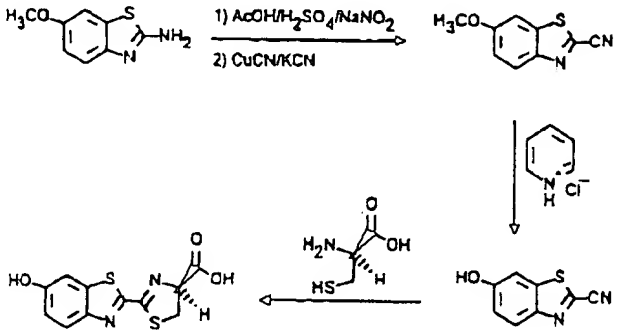
As had been assumed for a long time[88a, b] and to a large extent proven at the end of the 1970s,[88c] the specific luciferase of the firefly catalyzes the oxidation of luciferin in the presence of ATP and magnesium ions (Scheme 16). Initially a complex is formed from the acyl-AMP species of luciferin and luciferase. In the presence of oxygen oxidation ensues to give excited oxyluciferin which returns to the ground state by emitting a photon.[15, 89, 90] In vivo the yellow-green emission (λ_{max} = 565 nm) of the dianion was observed and in vitro an additional red emission (λ_{max} = 615 nm) of the monoanion which was pH-dependent.[88b, 90, 91] The oxidation proceeds presumably via a dioxetanone intermediate[11, 87, 92] which decarboxylates to furnish excited oxyluciferin.

To what extent one can view the often proposed dioxetanone as an intermediate or rather as a transition state is, as in the luminescent systems previously mentioned, still unclear. Instead of the dioxetanone intermediates in the oxidation of luciferins, acridiniumcarboxylic acid derivatives, and oxalic acid esters, the direct formation of excited products by charge-transfer can also be assumed during the decomposition of peroxide intermediates.[93] The mechanistic details cannot be emphasized within the framework of this review and interested readers should refer to the references [11, 87, 92, 93].

All in all, the light reaction of the firefly appears to be elucidated. However, the assumption, first made at the end of the 1950s,[94] that Coenzyme A also plays a role in the light reaction



Scheme 16. Chemistry of the light reaction of the firefly: PP = pyrophosphate; ATP = adenosine triphosphate, AMP = adenosine monophosphate, CoASH = coenzyme A.



Scheme 17. Key step in the synthesis of the firefly luciferin.

has been comfirmed in the past few years.[89, 95] Addition of the coenzyme may further improve the applicability of the firefly luciferin/luciferase system in the near future, since the intensity and duration of the light emission can be increased. The limiting factor for this method until now, in addition to the limited hydrolytic stability of luciferin and the sensitivity of luciferase, was, above all, the poor availability of the enzyme which could be extracted from fireflys. In the meantime the situation has fundamentally changed since the *Photinus Pyralis* luciferase, a protein with a molecular weight of 62 kD, can be expressed in bacteria, for example *E. coli*, by genetic engineering methods.[15, 89, 96, 97] The availability of genetically engineered luciferase and synthetic luciferin has now increased the expectation that this system, which with a quantum yield of up to 0.88 Einstein per mol is the most efficient of all known bio- and chemiluminescence systems, will be more widely applied than previously.

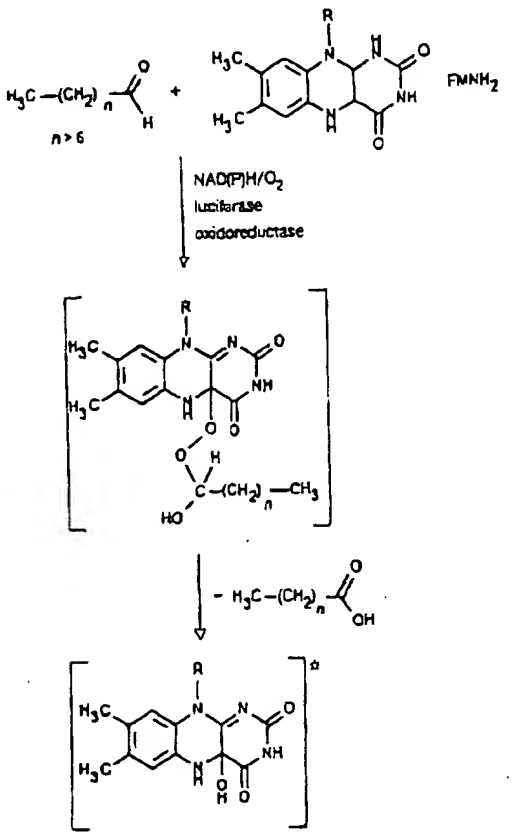
In the last thirty years essentially three synthetic pathways for the construction of firefly luciferin have been described, all of which proceed via the key intermediate 6-methoxybenzothiazole-2-carbonitrile (Scheme 17).[91, 98] The routes differ in the synthesis of the intermediate. Recently a new synthesis[99] has been published in which 6-methoxybenzothiazole-2-carbonitrile is obtained in one step by Sandmeyer cyanation of the commercially available 2-amino-6-methoxybenzothiazole (Scheme 17). The remainder of the synthetic pathway is already well-known: cleavage of the methyl ether and condensation with D-cysteine furnish the luciferin. In reference [99], earlier syntheses are also summarized. The oldest and until now most important application of the firefly luciferin/luciferase system is derived from the ATP-dependence of the bioluminescence reaction. Hence, a sensitive ATP determination can be carried out by using this system. ATP assays[100] are of interest above all in the screening for microorganisms in clinical microbiology in the areas of hygiene and nutrition.[101] A more recent application of steadily increasing significance is the use of the firefly luciferase gene as a reporter gene for the quantification of the gene expression in cells.[39, 102] Here a measurement of the light emission is made after addition of luciferin.

The use of luciferin derivatives,[103] which themselves are not substrates for luciferase, as substrates in enzyme immunoassays offers additional applications. After the luciferin has been released light emission is determined in the presence of luciferase.[103] In this way derivatives in which the phenolic hydroxyl group has been functionalized, such as D-luciferin-*O*-sulfate and -*O*-phosphate, can be cleaved by sulfatases or phosphatases. If the carboxyl group of the luciferin can be functionalized (methyl ester and phenylalanine and arginine amides have been described), the luciferin is released by carboxyesterases or carboxypeptidases.[103] Luciferin-*O*-phosphate as substrate for alkaline phosphatase, in comparison to the chromogenic substrate *p*-nitrophenylphospate, facilitates a sixty-fold increase in sensitivity.[103a] In addition, D-luciferin-β-D-galactopyranoside as substrate for β-galactosidase was described.[104]

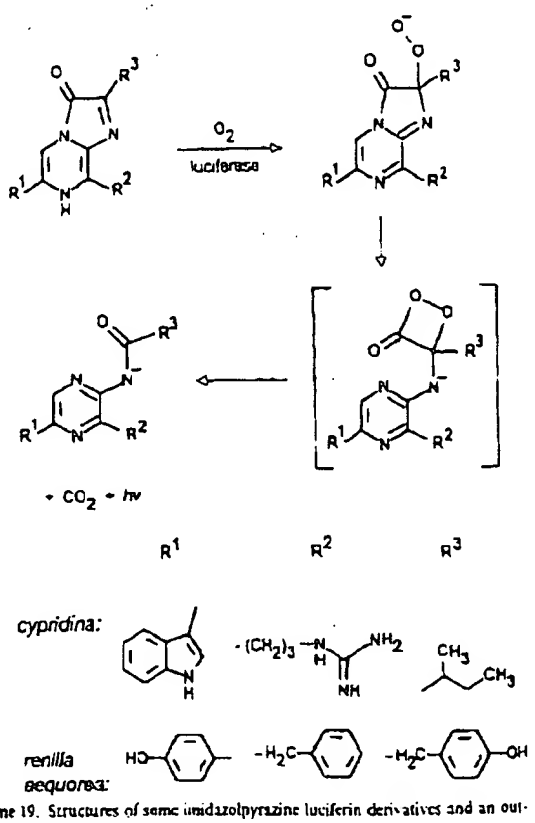
Bacterial luciferin derivatives/luciferases

In luminous bacteria such as *Photobacterium fischeri* and *Photobacterium phosphoreum*, light production ensues from the oxidation of long-chain aldehydes in the presence of reduced flavin mononucleotide ($FMNH_2$), oxidoreductase, and bacterial luciferase.[15, 90, 97, 105] The intermediate is assumed to be a peroxide formed from a long-chain aldehyde and a flavin building block (Scheme 18). Depending on the bacterium, light emission occurs in the blue-green to yellow region of the spectrum with quantum yields of up to 0.3. The emitter is presumably a hydroxy derivative of FMN.[11, 13, 103] In vitro blue light (λ_{max} = 492 nm) is emitted.[15, 105a] As with the firefly luciferase, several bacterial luciferases can be obtained by genetic engineering.[97]

The bioluminescence of bacterial luciferases can, in principle, be used to determine all the components that participate in the luminescence reaction, that is, NADH, NADPH, FMN, $FMNH_2$, long-chain aldehydes, and oxygen.[105a] The possibility of determining the concentration of the extremely unstable $FMNH_2$ is theoretical. The use of bacterial luciferases is indeed still less widespread. The bioluminescent determination of long-



Scheme 18. Bacterial light systems.



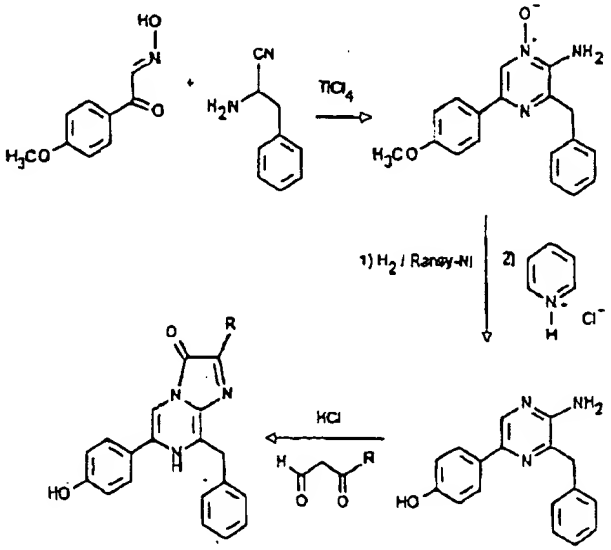
Scheme 19. Structures of some imidazolpyrazine luciferin derivatives and an outline of the peroxylactone mechanism.

chain aldehydes[105] and trace analysis of oxygen[105c] have some significance. A further field of application is in homogeneous DNA hybridization assays.[15, 107] The system for the bioluminescent determination of Papilloma viruses serves as an example.[107]

Luciferin derivatives with imidazopyrazine building blocks and photoproteins

Bioluminescence occurs particularly frequently in marine life such as in crabs, jellyfish, mussels, sponges, fungi, and many fish.[9, 92b, 109] The luciferins of the mussel crab *Cypyridina hilgendorfii*, sea pansy *Renilla reniformis*, and of the jellyfish *Aequorea aequorea* show a structural similarity (Scheme 19) which infers a common biosynthetic pathway.[92a] The luminescence mechanism is thought to consist of a catalytic oxygenation followed by ring closure to give an α-peroxylactone. This intermediate decomposes with the formation of the emitter and carbon dioxide (Scheme 19). One should refer to the relevant discussions of the theories concerning the firefly luminescence mechanism in specialist literature.

The photoprotein Aequorin, which was obtained from the jelly fish *Aequorea victoria* in 1962, has aroused particular interest in recent years.[109] It consists of a complex of apoaequorin, coelenterazine (cf. Scheme 19), and molecular oxygen. Addition of calcium or strontium ions to the complex triggers light emission.[110a] One assumes that the binding of calcium ions to the protein induces the decomposition of the resulting oxygenated chromophore. An additional luciferase is not necessary. The emitter was postulated to be the protein-bound anion of the chromophore (cf. Scheme 19).[110b] The active photoprotein is regenerated by incubation of the apoprotein with the coelenterazine in the presence of oxygen, ethylenediaminetetraacetate, and mercaptoethanol.[110c] In the meantime the apoprotein has become accessible by expression in *E. coli*.[111] The synthesis of the coelenterazine was described long ago,[112a, b] and in addi-



Scheme 20. Synthetic route for the construction of coelenterazines. R = CH_3, C_6H_5, $(CH_2)_4C_6H_5$ ($n = 1-3$), $CH_2C_6H_4OH$.

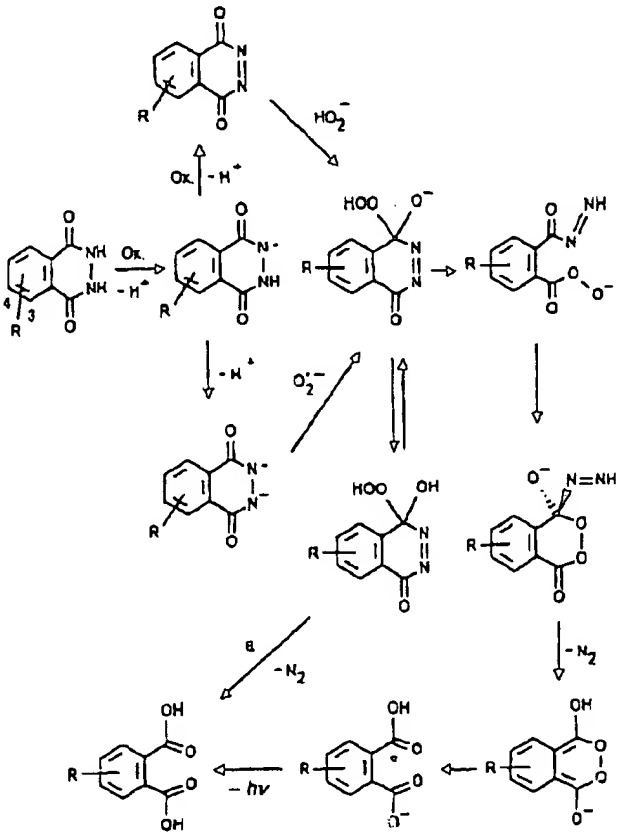
tion the synthesis of some systems with a modified structure was reported.[113c] The synthetic route is summarized in Scheme 20.

Aequorin can be used as a bioluminescent label after biotinylation. The triggering of light emission results from addition of calcium chloride solution. The label can also be detected in the attomol region. This label facilitated, for example, the development of a highly sensitive assay for salmonella determination. With regard to the sensitivity the test was shown to be clearly superior to other ELISA tests (see Section 3.1.2), even those with alkaline phosphotase as label and with chemiluminescent dioxetane AMPPD as substrate.[113a,b] In addition, the use of Aequorin in DNA and protein diagnostics[113c] and in the determination of serum glycoproteins has been described.[113d]

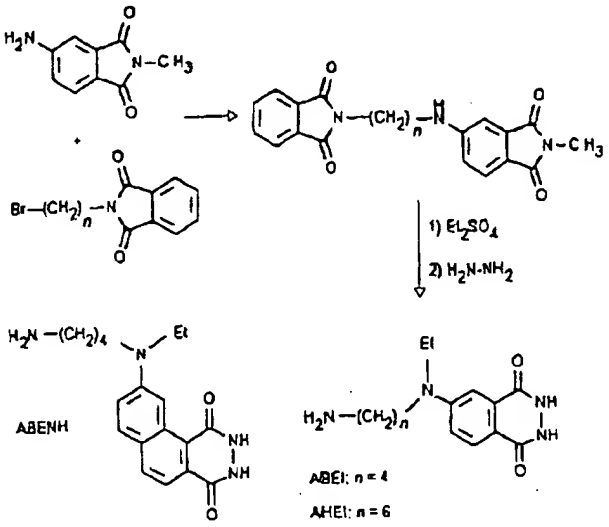
5.2.2. *Cyclic Arylhydrazides*

The chemiluminescence of luminol (3-aminophthalic hydrazide) was observed in 1928 in the form of blue light which was emitted during the oxidation with an alkaline solution of hexacyanoferrate(III) in the presence of hydrogen peroxide.[114] Luminol and carboxylic acid hydrazides have since been extensively studied. A wealth of reagents and catalysts can be employed in the oxidations of luminol and its derivatives. In organic, aprotic solvents, chemiluminescent reactions can be triggered by oxygen in the presence of a strong base. In aqueous solutions hydrogen peroxide is usually employed in the presence of catalysts such as peroxidases, hemin, and cobalt(II) salts.[105] Horseradish peroxidase (HRP)/H_2O_2 is frequently employed. In the course of time, different reaction mechanisms have been discussed.[45a] A simplified reaction mechanism, which is only applicable for a one-electron oxidation and which affords the free luminol radical, is given in Scheme 21. According to a more recent review article it is suggested that the mechanism should be divided into two steps, formation of the key intermediate, an α-hydroxyhydroperoxide, and its decomposition to the excited emitter. Whilst the formation of the hydroperoxide depends considerably on exact reaction conditions, decomposition of the key intermediate is only influenced by pH. Under these conditions the emitter appears to be the monoanion of aminophthalic acid. With other reagents, for example DMSO/base, the dianion of aminophthalic acid functions as the emitter. Intermediates in other proposed mechanisms are azaquinones, endoperoxides, and other peroxidic intermediates.[115b]

One of the oldest applications of luminol, which is still important today, is forensic blood analysis.[116] The application of luminol as a substrate for peroxidases in enzyme immunoassay has already been mentioned. Coupling reactions are necessary for the synthesis of labels for chemiluminescent direct labeling. The study of the chemiluminescent properties of luminol and isoluminol derivatives[117] had revealed that isoluminol only has approximately 10% of the quantum yield of luminol. Since, however, the chemiluminescent quantum yield of luminol decreases significantly[25, 37, 115, 117] if the primary 3-amino group is substituted, isoluminol derivatives, which are not sensitive with regard to substitution of the amino group, or the even more advantageous naphthalenedicarboxylic acid hydrazides which exhibit higher light yields,[21, 117b] were as a rule employed in the synthesis of arylhydrazide labels. The first label based on luminol, diazoluminol,[118] clearly showed the disadvantages outlined and the quantum yield decreased to 1% of that of luminol. Considerable improvements were brought about by isoluminol derivatives in which the coupling group was introduced via the amino functionality.[117b] The structure and the synthesis of some important arylhydrazide labels are summarized in Scheme 22.



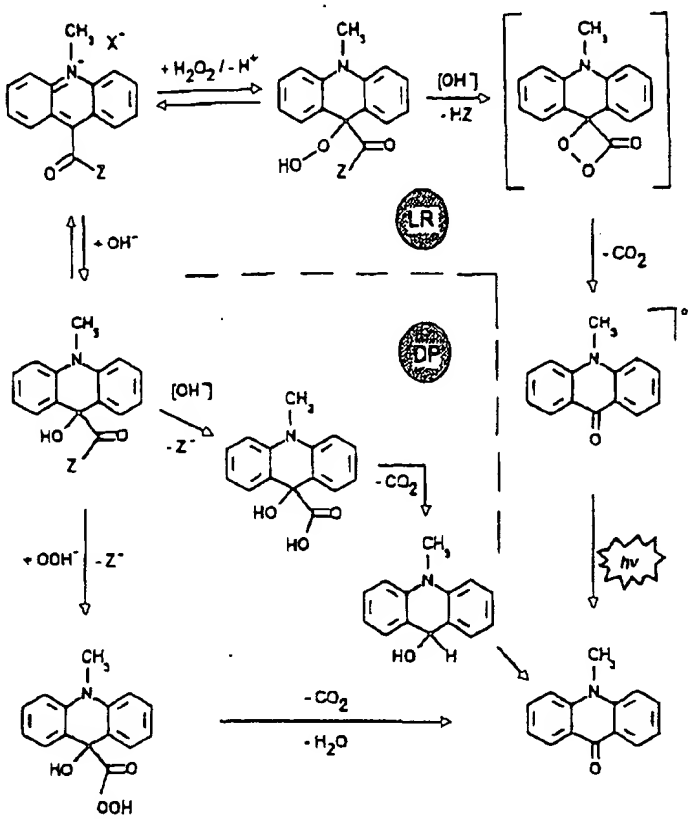
Scheme 21. Simplified mechanism of the chemiluminescence reaction of phthalic hydrazides. Ox = oxidising agent which affords the free luminol radical, for example, HRP. R = 3-NH_2: luminol; R = 4-NH_2: isoluminol. Since the regiochemistry of most processes is unclear, the position of the R residue remains open. a = dark.



Scheme 22. Synthesis and structures of some arylhydrazide labels.

4-Amino-*N*-methylphthalic imide served as the starting material, which was successively alkylated with *N*-(bromoalkyl)phthalic imides and diethylsulfate. The subsequent hydrazinolysis of the bisphthalic imide furnished the frequently used phthalic hydrazide labels ABEI and AHEI (*N*-aminobutyl- or *N*-aminohexyl-*N*-ethylisoluminol), which contain an amino functionality as the coupling group. The phthalic hydrazide label ABENH (*N*-aminobutyl-*N*-ethylnaphthylhydrazide) was obtained by a similar method. The starting material was dimethyl 7-amino-1,2-naphthalenedicarboxylate.[117b] Derivatives of these three labels which contain other reactive groups (isothiocyanate, N-hydroxysuccinimide ester) are also known. Conversion of AHEI to a derivative with a *N*-hydroxysuccinimide-reactive ester was shown previously in Scheme 3.

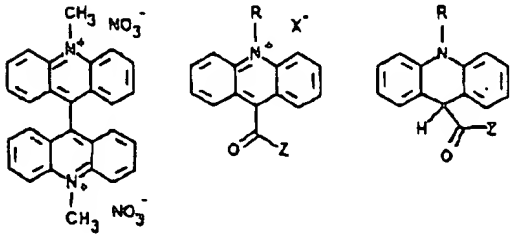
Arylhydrazide labels have and are finding broad application in immunoassays for chemiluminescent labeling of small and large molecules.[119] A distinct disadvantage is the considerable loss of the luminescence quantum yield of the label after the coupling. Furthermore, these labels are prone to interference since many components catalyze the luminescent reaction. In addition, the reagents which trigger light emission give rise to a large background signal which decreases the sensitivity.[25]



Scheme 24. Chemiluminescence mechanism and pseudo-base equilibrium of 9-acridiniumcarboxylic acid derivatives: DP = dark process, LR = light reaction.

5.2.3. *Acridinium Compounds*

The chemiluminescence of lucigenin [9,9'-bis-(*N*-methylacridinium nitrate); Scheme 23] was reported in 1935.[120] But it was



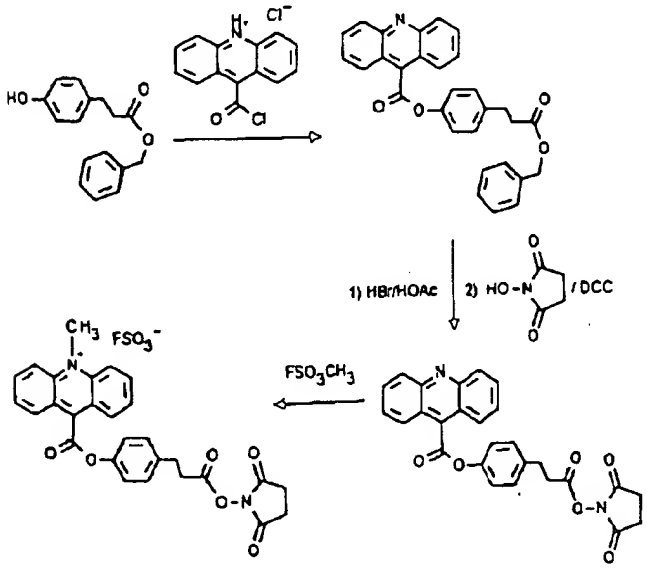
Scheme 23. Examples of chemiluminescent acridinium compounds and acridanes; Z = leaving group.

not until around thirty years later that studies led to other chemiluminescent acridinium compounds. These are specifically, 9-chlorocarbonylacridine hydrochloride,[121] 9-carboxy-10-methylacridinium chloride,[121] and 9-cyano-10-methylacridinium nitrate.[122] Today 9-acridiniumcarboxylic acid derivatives and acridanes are among the best-studied examples of chemiluminescent compounds. No additional reagents, apart from hydrogen peroxide and base, are necessary for the chemiluminescence of acridiniumcarboxylic acid derivatives. Quantum yields of up to approximately 0.05 can be attained with aryl esters;[111] higher still are the light yields which can be attained with acridane aryl esters. The latter exhibit efficient chemiluminescence after treatment with a base in the presence of oxygen.[123] Only the acridiniumcarboxylic acid derivatives that have been more important for the development of luminescent labels will be considered in more detail below. The mechanism of their chemiluminescence shown in Scheme 24 can be considered to be elucidated as far as possible.

First of all, addition of hydrogen peroxide takes place at the electrophilic C-9 position of the acridinium unit. In the case of aryl esters—the leaving group Z then stands for phenolate—the corresponding hydroperoxides could be isolated and characterized.[123] After the addition of hydroxide spontaneous chemiluminescence resulted, usually as intense light flashes. A dioxetanone is often proposed as intermediate, which decomposes to give carbon dioxide and electronically excited *N*-methylacridone, the emitter. The transition to the ground state ensues by emission of a photon at a wavelength of approximately 430 nm. According to more recent studies,[93b] however, no dioxetanone seems to appear as a discrete intermediate. The final product of the light reaction, *N*-methylacridone, can also be formed by other pathways in dark reactions. An important prerequisite for degradation in the dark is the well-known[124] pH-dependant pseudo-base equilibrium of acridinium compounds.[125] The reactions, which for the phenyl ester were studied more accurately in a flow system,[126] are integrated into Scheme 24. It is immediately clear that both the light reaction and the dark process are dependent, for instance, on the properties of the leaving group Z. Other important factors include the peroxide concentration

and the pH value.[126, 127] McCapra et al. showed that for effective chemiluminescence the pK value of the conjugate acid of the leaving group should be less than 12—the pK value of hydrogen peroxide.[108c] Since a good label should exhibit a high chemiluminescent yield in addition to a high stability in the labeled reagent, the discovery of suitable compounds can be equated to a fine balancing act, ending in a compromise between light yield and stability.

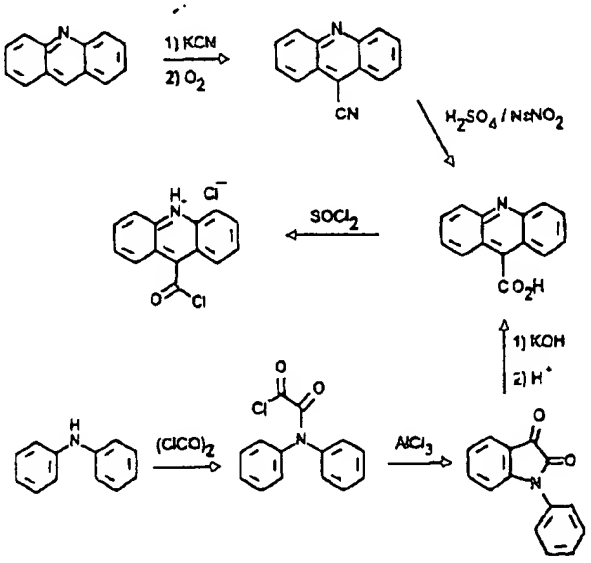
The first attempts to use 9-acridiniumcarboxylate as a label in immunoassays were reported at the beginning of the 1980s. In this an attempt was made to couple aryl esters, which contained free carboxyl groups, to proteins, after activation of the carboxyl groups. These labeling experiments had only limited success.[128] It was not until a phenyl N-methylacridinium-9-carboxylate, containing a hydroxysuccinimide ester on the phenyl group as the reactive group for coupling to proteins, was used that successful labelings could be carried out.[126, 129] The synthesis of this prototype of a chemiluminescent label based on a 9-acridiniumcarboxylic acid derivative,[25, 126, 128–130] the so-called Woodhead label, is summarized in Scheme 25.



Scheme 25. Synthesis of an acridinium ester label.

An important precursor for this and other labels discussed later was 9-acridinecarboxylic acid, whose synthesis is shown in Scheme 26. One reaction route starts from acridine and proceeds via 9-cyanoacridine to give the carboxylic acid.[131] In another synthetic method 9-acridinecarboxylic acid is formed from diphenylamine, which is acylated with oxalyl chloride and is cyclized by using aluminum trichloride to give the N-phenylisatine.[132] The synthesis of substituted acridinecarboxylic acids from substituted N-arylisatines has also been described;[133] however, it appears to work only on a very small scale.

Since the acridinium ester label mentioned was not sufficiently stable for development of commercial chemiluminescence immunoassays[134, 135] (hydrolysis of the ester bond (cf. degradation in the dark in Scheme 24) results in a too rapid a decrease of activity in conjugates), different research groups have been



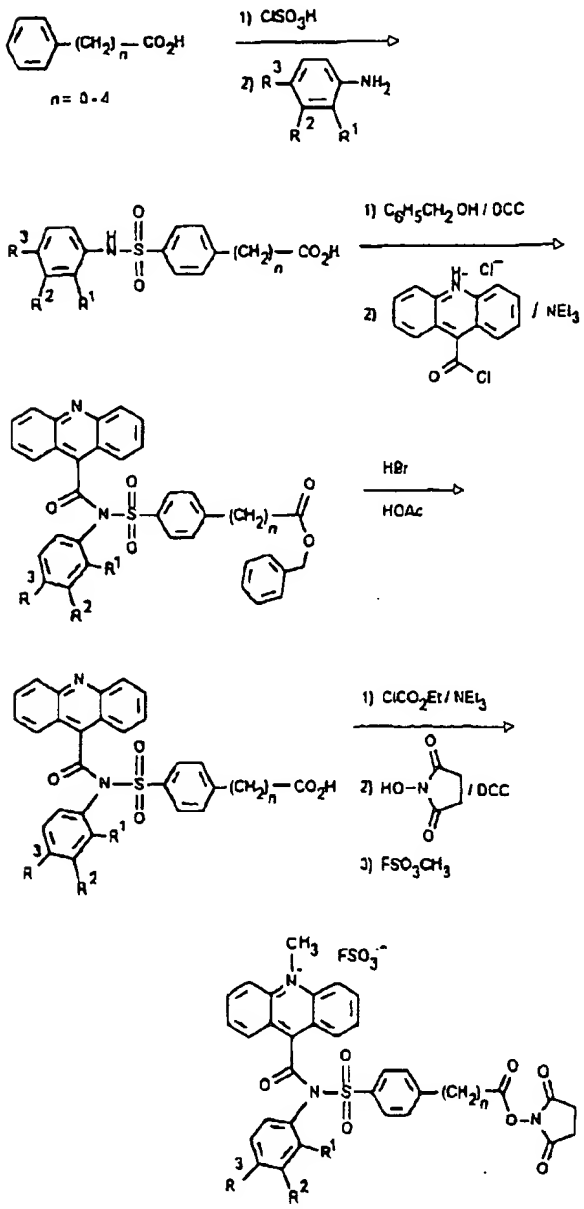
Scheme 26. Possible synthetic routes to 9-acridinecarboxylic acid.

looking for more stable labels based on acridiniumcarboxylic acid. One solution to the problem involved the steric shielding of the ester bond and the C-9 position of the acridine unit, for example, by methyl groups in the 2,6-position of the aryl ring.[134] In addition, other 9-acridiniumcarboxylic acid-(2,6-substituted)aryl esters were described.[136] Here the reactive group can also be bound through a spacer to the acridinium system. To complete the picture one should not forget to mention that aryl N-methylphenanthridinium-6-carboxylates have been described as chemiluminescent labels.[136, 137]

Other research groups have attempted to improve the properties of the acridiniumcarboxylic acid derivatives in comparison to those of the aryl esters, by varying the leaving groups. Thiol esters, indeed, brought about progress in as far as the light yield is concerned, but not, however, as regards to hydrolytic stability.[138] A significant improvement of the stability and very good chemiluminescence quantum yields were achieved when N-sulfonylamide anion was used as the leaving group instead of phenoxide.[138–141] In this class of compounds the spectrum of properties can be influenced much more specifically than for the acridinium ester labels by tailored variations in the structures. For instance, the solubility in water (an important parameter) can be significantly improved by the introduction of suitable substituents.[139, 142] The synthesis of N-methylacridinium-9-(N-sulfonyl)carboxamide labels is summarized in Scheme 27.

In addition, the hydrolysis behavior and the kinetics of emission can be varied to a certain extent.[142, 143] Although phenols and sulfonamides have similar pK values, labels with the latter are usually considerably more stable. This may be attributed to a combination of steric shielding effects and electronic stabilization,[127, 143] through which the dark reaction pathways described are minimized. It is assumed that in N-sulfonylcarboxamides there is an increased bond order of the C–N bond in comparison to the ester C–O bond. This is also evident from the frequencies of the carbonyl stretching vibration in the IR spectrum.[143]

Labels based on 9-acridiniumcarboxylic esters and acridinium-9-(N-sulfonyl)carboxamides have, in the meantime, found

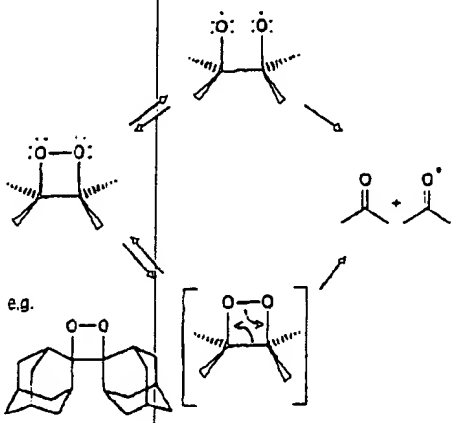


Scheme 27. Synthesis of acridinium-9-(*N*-sulfonyl)carboxamide labels.

broad application in commerical immunoassays (see Section 6).[119c, 144] Up until now only a few applications for other acridinium derivatives have been described. For example, lucigenin can be used in micellular chemiluminescence assays for the determination of reductants (ascorbic acid, uric acid, glucose, and fructose).[145] The micelles are necessary to improve the solubility of lucigenin.

5.2.4. Dioxetanes

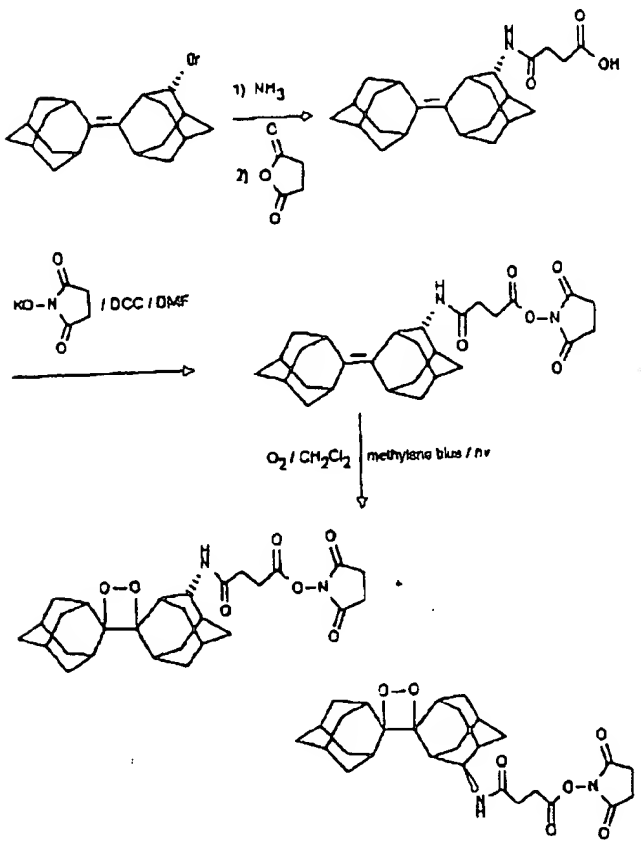
Dioxetanes have for a long time been regarded as merely having curiosity value in the laboratory. Use of the extremely unstable compounds in reagents for diagnostics was not considered. It was not until after adamantylideneadamantane-1,2-dioxetane (an extremely stable compound due to the steric shielding prepared by W. Adam et al.[146] in 1972) became known that the development of dioxetane labels really took off. Light emission could only be triggered thermally for stable dioxetanes of the type mentioned which exhibit a half-life of greater than 20 years at room temperature. In the thermolysis two molecules of adamantone are formed, partly in the S_1 state and partly in the T_1 state (Scheme 28). In principle, cleavage can occur according



Scheme 28. Simplified representation of the decomposition reaction of dioxetanes.

to a diradical or a concerted mechanism.[87] A stepwise pathway, involving homolysis of the O–O bond and formation of a diradical, has been proposed for the decomposition of the stable dioxetanes.[147] The light emission results from the deactivation of the S_1 excited species. Dioxetanes, which, in addition to the steric stabilization by only one adamantyl group, still contain a substituent of low oxidation potential, mostly aryloxy, undergo a different decomposition mechanism. This decomposition route is triggered by cleavage of the O–O bond and by an electron transfer from the oxidizable group into the antibonding orbital of the peroxide bond (CIEEL mechanism, chemically initiated electron exchange luminescence).[92c, 148–155] This mechanism for the dioxetanes mentioned which are substrates for enzyme labels is discussed in Section 4.

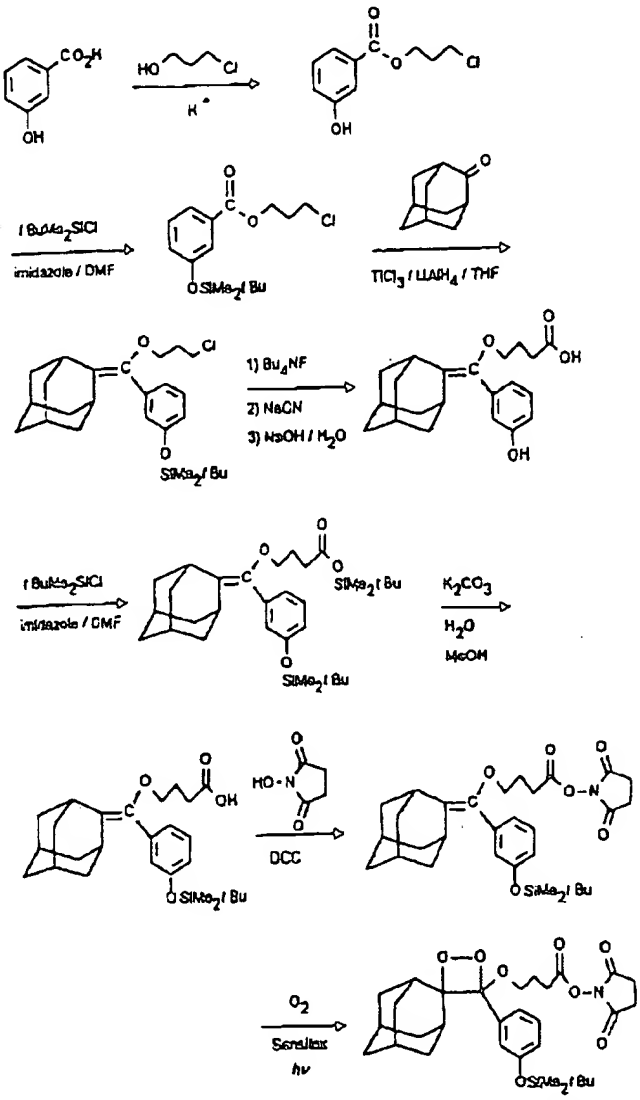
Functionalized adamantylideneadamantanes that contain a reactive group bound to a spacer have been described as a label for thermochemiluminescent immunoassays[156] (Hummelen et al., 1986). The synthesis of one such label is given in Scheme 29. The starting material is adamantylideneadamantane, which can be obtained in two steps from adamantanone.[157] In the last step of the synthesis, sensitized (methylene blue) photooxygenation, a mixture (ca. 1:1) of two dioxetane isomers results, which was used in this form in the labeling experiments. The overall yield of the seven-step synthesis, starting from adamantanone is 50%. The triggering of luminescence results from heating the sample adsorbed onto aluminum oxide for a short time at 240 °C. An apparatus for measuring thermochemiluminescence has also been described.[156] Since the efficiency of the direct chemiluminescence of adamantylideneadamantane-1,2-dioxetane is 1×10^{-4} (6×10^{19} photons per mol)[158] under optimal conditions (only 1% of that of luminol), an increase in the energy transfer to a good fluorescent dye is necessary. Bovine serum albumin conjugates with the dioxetane label and 9,10-diphenylanthracene, which, in turn, have been used as labels in



Scheme 29. Synthesis of a thermochemiluminescent dioxetane label. DMF = dimethylformamide.

*f*luorescence *a*mplified *t*hermochemiluminescence *i*mmuno*a*ssay (FATIMA), have been described. The first assays, for example, for the tumor marker CEA have been described, but the thermochemiluminescence principle appears to be altogether too costly for wider commercial use. The search was then started for model compounds for thermally somewhat less stable dioxetane labels, which could be activated at approximately 150 °C.[159] For example, 9-xanthenylideneadamantane decomposes at around 100 °C (Hummelen et al., 1988). The light emitted from this compound corresponds to the emission from adamantone. Other monoadamantyldioxetane derivatives of the xanthene, naphthalene, and phenyl series, which can be triggered enzymatically and chemically and in which the olefinic starting material contains a methoxy substituent to facilitate dioxetane synthesis, have been described (Schaap et al., 1987).[47c, 47d] Shortly afterwards AMPPD, already mentioned in Section 4, was reported. In contrast to the above-mentioned thermal decomposition, the emitter in the CIEEL decomposition of adamantylidenearyloxy-1,2-dioxetanes, which can be triggered enzymatically or chemically (cf. Scheme 10), is chiefly an excited aryloxy anion.

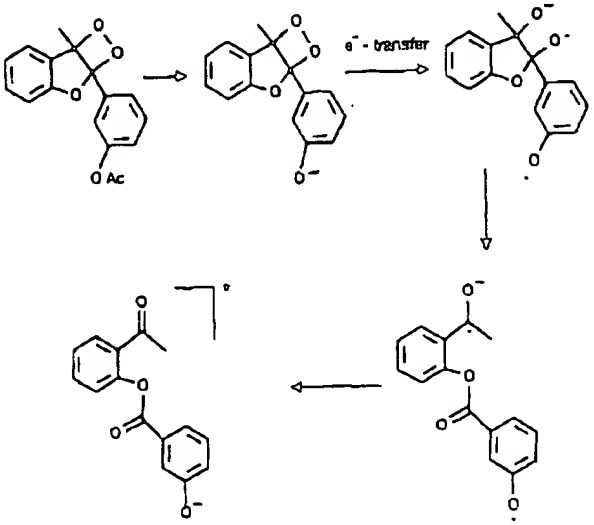
Thus, in less than twenty years since their discovery, dioxetanes have developed from merely being laboratory curiosities to being stable derivatives employed worldwide in immunolgical and biochemical analysis. The development has not yet reached an end. Until a short time ago the 1,2-dioxetanes, which can be triggered enzymatically or chemically, were not known as classical labels with a reactive group for coupling to molecules to be labeled, however, recently also such dioxetane labels for direct labeling methods were introduced.[14, 47a, 160] The synthesis of a selected label is outlined in Scheme 30. Apart from the silyloxy



Scheme 30. Dioxetane labels for chemiluminescent direct labeling which can be triggered chemically. Sensitox = polymer-supported Rose Bengal.

group shown which can be cleaved to trigger luminescence, other substituents can also be employed, for example, phosphate and galactosyl groups. In addition to the hydroxysuccinimide esters shown other common reactive groups should also be considered. A label with a biotin anchor and its use for labeling proteins and antibodies has also been described.[47b] Further applications of this relatively new label are as yet unknown, but judging from the high quantum yields (0.20–0.25 in DMSO)[14] they will presumably not be long in coming.

Finally in this section on dioxetanes another new class of relatively stable dioxetanes should be mentioned namely, phenyl-substituted benzofuran-1,2-dioxetanes.[14, 92a–f] These give quantum yields (up to 4×10^{-6}) similar to those of the enzyme substrates described earlier. The acetoxy-substituted



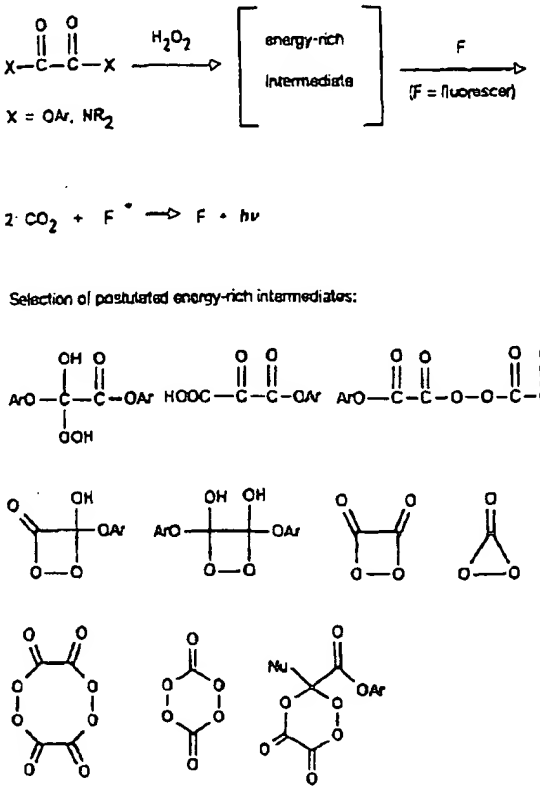
Scheme 31. Structure and decomposition of a benzofuraadioxetane.

compound shown in Scheme 31 decomposes, presumably base-induced, according to the CIEEL mechanism. For analogous siloxy-substituted compounds decomposition can, as with similar adamantyl systems, be triggered by fluoride ions.[92f]

5.2.5. Oxalic Acid Derivatives

Oxalic acid derivatives such as oxalyl chloride,[161, 162] certain oxalic acid anhydrides,[163] diaryl esters,[164] and oxamides[165] are among the synthetic molecules which exhibit the highest chemiluminescence quantum yields (up to 0.5).[11] Intense luminescence is observed in the presence of a fluorescent dye during the oxidation of oxalic acid derivatives with hydrogen peroxide. Despite extensive studies the complete mechanism of peroxyoxalate chemiluminescence is still not fully understood. A dioxetanedione is often proposed as the energy-rich intermediate which forms the oxalic acid derivative by reaction with hydrogen peroxide (Scheme 32). However, according to the results of more recent work, this highly strained intermediate is not formed;[93b] a large number of possible intermediates is shown in Scheme 32.[14, 166–169] As has already been considered in other mechanisms, reaction pathways involving electron transfer or energy transfer are discussed.[14] Finally, either excited carbon dioxide is formed which activates the fluorescent dye by energy transfer, or a charge-transfer complex is formed which decomposes to give CO_2 and a fluorescent dye molecule in the excited state. The luminescence of these systems is relatively long lasting and the emission color can be controlled by choice of dye. The best known application of this are the Cyalume light sticks.[170]

Oxalic acid esters or oxamides cannot be considered for the development of luminescent labels for diagnostic purposes because the solubility of the oxalates and fluorescent dyes requires the use of organic solvents; the compounds are hydrolyzed quickly in aqueous solutions.[171] However, oxalates are employed in chemiluminescence detectors in HPLC or flow injection analysis.[14, 171] The appropriate systems allow, inter alia, analysis of environmental toxins, drugs, amino acids, fatty acids, and amines with detection sensitivities ranging from the nanogramm



Scheme 32. Mechanism of chemiluminescence for oxalic acid derivatives.

into the attomol region. Another interesting area of application of oxalate chemiluminescence in diagnostics is the quantitative determination of oxalic acid in urine with detection limits as low as 10 nmol L^{-1}. For this purpose the oxalic acid present is treated with carbodiimide in the presence of hydrogen peroxide and a fluorescent dye. The light emission measured is proportional to the concentration of oxalic acid.[172, 173] Even the determination of porphyrins in urine is possible with this method. In this case a fluorescent dye is not necessary because the porphyrins themselves act as sensitizers.[174]

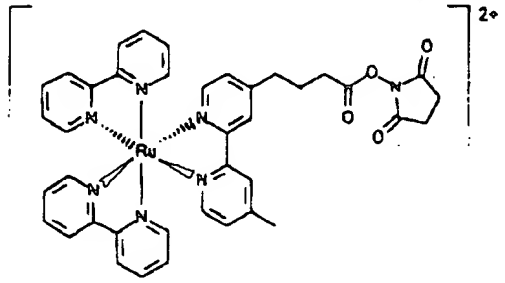
5.2.6. Electrochemiluminescence

The triggering of chemiluminescence by electrochemical processes has been known for a long time but has only gained practical significance in recent years.[175] Radicals produced electrochemically play a significant role in these processes and the electrochemical excitation of luminol/hydrogen peroxide mixtures has been studied in detail.[176] Luminescence is observed with potentials greater than 0.5 V. A mechanism similar to the one presented in Scheme 21 is assumed for potentials of up to 0.7 V. Diazaquinone produced electrochemically reacts with hydrogen peroxide. At higher potentials (1.2 V) the reaction is considerably more complex since the amino group of luminol is involved in oxidation processes.[176] An electrochemical detector based on this system can be used for the determination of hydrogen peroxide.

In addition, the electrochemical processes of acridinium compounds have been investigated.[177] Whilst lucigenin is reduced at −0.3 V and gives rise to luminescence in a subsequent reaction of the radical, acridinium esters show no activity in the range +1

to 1 V. However, the luminescent reaction of the acridinium esters can be initiated electrochemically by hydrogen peroxide produced from oxygen. This principle facilitated development of detectors for labeled analytes without the need for additional reagents. Lysin labeled with the first acridinium ester described earlier could be detected with a sensitivity of 10 fmol.[177]

The chemiluminescence of ruthenium(II) chelate complexes has been known for a long time,[178a, 179a] likewise the electrochemically generated luminescence from trisbipyridineruthenium(II) chelates.[178b] However, the labeling of haptens, proteins, and nucleic acids with ruthenium(II) chelates was only described recently.[179a] The ruthenium complex, which is shown in Scheme 33, uses a hydroxysuccinimide ester sub-



Scheme 33. Structure of an electrochemiluminescent label based on a [trisbipyridine]ruthenium chelate

stituent as the reactive group. The advantages of the label cited are high stability, relatively low molecular weight, high solubility in water, and high sensitivity – the detection limit of the label is 200 fmol.[179a] Multiple labeling of proteins and oligonucleotides are possible without being detrimental to immune reactivity, solubility, or ability of the conjugate to hybridize. In the electrochemical reaction $[Ru(bpy)_3]^{2+}$ (bpy = bipyridine) is first oxidized to $[Ru(bpy)_3]^{3+}$ on the electrode surface. Simultaneously, the tripropylamine (TPA) present in a large excess is likewise oxidized to a radical cation $TPA^{+\cdot}$ which spontaneously cleaves a proton. In the reaction of the strong oxidizing reagent $[Ru(bpy)_3]^{3+}$ with the radical $TPA^{\cdot}$, a strong reducing agent, a $[Ru(bpy)_3]^{2+*}$ complex is formed in the electronically excited state which returns to the ground state by emission of a photon at 620 nm. The ruthenium(II) complex can re-enter the cyclic process, which automatically causes amplification of the signal.

In addition to an electrochemiluminescent analyser,[179b] immunoassays,[179a] and genetic probe tests[179a, 180] with the, in the meantime, commercially available ruthenium label have been described. The available data is insufficient to evaluate the suitability of electrochemiluminescent detection in diagnostic practice.

6. Applications

6.1. Immunoassays

6.1.1. Introduction

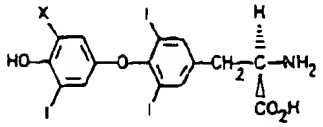
When Yalow and Berson developed the first radioimmunoassay for the in vitro determination of insulin in 1959,[1] they surely could not have envisaged that their method in this or in some other form would become the most important analytical tool of medicinal in vitro diagnostics. It is difficult to overlook the wealth of substances, whose concentrations are today routinely determined in clinical laboratories with immunoassays. A number of these analytes are listed in Table 2 according to diagnostic indications.

Table 2. Some analytes which are routinely determined by immunoassay in different diagnostic areas.

Diseases of the thyroid gland	*Gastrointestinal tract*
TSH (thyroid stimulating hormone, thyrotropin)	calcitonin
T3 (triiodothyronine)	C-peptide
FT3 (free T3, i.e. not bound to binding protein)	gastrin
T4 (tetraiodothyronine)	insulin
FT4 (free T4, i.e. not bound to binding protein)	anti-insulin antibody
TBG (thyroxine binding globulin)	pepsinogen
Tg (thyreoglobulin)	trypsin
Anti-Tg (autoantibody against Tg)	trypsin neonatal
TPO (thyroidal peroxidase)	vitamin D
Anti-TPO (autoantibody against TPO)	
TRAK (autoantibody against the TSH receptor)	*Hypertonia and nephrology*
	aldosterone
Tumour growth controls	androstendione
AFP (α-fetoprotein)	angiotensin I (renin)
CA 50 (CA = cancer antigen)	cortisol
CA 125	DHEA (dehydroepiandrosterone)
CA 15-3	17 α-OH-progesterone
CA 19-9	
CA 72-4	*Cardiology*
CA 242	digoxin
calcitonin	myoglobin
CEA (carcinoembryonic antigen)	
cyfra 21-1	*Connective tissue diseases*
ferritin	luminin
HCG (human choriongonadotropin)	NC1 (N-terminal collagen I)
β_2-microglobulin	PIIIP (procollagen-III-peptide)
NCAM (neural cell adhesion molecule)	7S-collagen
NSE (neuron specific enolase)	
osteocalcin	*Infectious diseases*
PAP (prostatic acid phosphatase)	HBsAg (hepatitis B surface antigen)
prolactin	HSV antigens
PSA (prostatic specific antigen)	p24 antigen (HIV antigen)
SCC (squamous cell carcinoma antigen)	rotavirus antigen
TATI (tumor-associated trypsin inhibitor)	antibodies against:
Tg (thyreoglobulin)	- FSME virus
TPA (tissue polypeptide antigen)	- HBsAg
	- HIV1
Pregnancy	- HIV2
AFP (α-fetoprotein)	- HSV
estriol	measles virus
HCG (human choriongonadotropin)	- rotavirus
progesterone	- rubella virus
	- toxoplasma gondii
Sexual functions	- varicella Zoster
FSH (follicle stimulating hormone, follitropin)	
LH (luteinizing hormone, lutropin)	*Inflammatory processes*
progesterone	α-antitrypsin
17α-OH-progesterone	CRP (C reactive protein)
prolactin	α_1-macroglobulin
testosterone	α_1-acid glycoprotein (Orosomucoid)

The success of immunological assays is owed primarily to their high specificity and sensitivity; antibodies, which are employed in immunoassays as detection reagents, can "recognize" at the molecular level smallest structural differences (much cited "lock-and-key" principle). For example, an antibody raised

against the thyroid hormone thyroxin binds with high affinity (equilibrium constants usually are of the order of 10^{10}–10^{12} L mol^{-1}), whereas triiodothyronine which differs by only one iodine atom is not recognized (Scheme 34).



Scheme 34. Structure of the thyroid hormones T3 (X = H, 3,3',5-triiodo-L-thyronine) and T4 (X = I, 3,3',5,5'-tetraiodo-L-thyronine, L-thyroxine).

As a result of this impressive specificity practically all substances with a molecular weight of greater than 100 D even in complicated liquids such as serum can be determined exactly without prior separation of similar substances. Modern labels can even be traced into the attomolar range (1 amol = 10^{-18} mol). Thus, by labeling antibodies with such labels, the substances to be analyzed can be quantified exactly to the femtomolar range (1 fmol = 10^{-15} mol). A current listing of almost 500 literature references can be found in "Bioluminescence and Chemiluminescence Literature - Immunoassays and Blotting Assays" by O. Nozaki et al.[101]

6.1.2 Categories

Immunoassays can be divided into different groups.

Group I: Competitive immunoassays with analyte tracer:

This group concerns assays in which the detection reagents are an antibody specifically directed against the substance to be determined and an analyte derivative which carries the label (analyte derivative tracer, usually abbreviated to analyte tracer). The analyte tracer should not be too structurally different from the analyte (which under the circumstances only exists in the presence of the label) that it no longer is recognized by the antibody. Furthermore, immunoassays of this group are competitive assays, that is the analyte and analyte tracer compete for a small number of antibody binding sites in an equilibrium reaction (Fig. 3a). The lower the concentration of the analyte-sample to be analyzed, the more antibody–analyte tracer complexes can form as a result. The analyte concentration of an unknown sample can be determined exactly by using a calibration curve drawn up from samples of known concentration (Fig. 3b). As is apparent from Figure 3a, selective measurement of the signal emitted from the antibody–analyte tracer complex requires prior separation of uncomplexed analyte tracer. Separations of this kind are dealt with in Section 6.1.3.

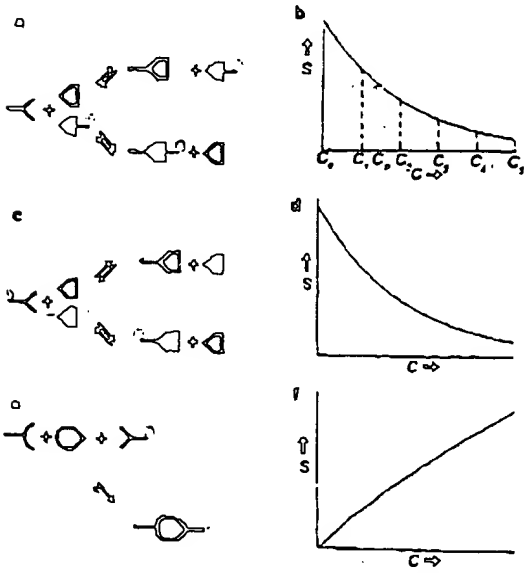


Fig. 3. Principle of the three most important immunoassay methods (left) with the corresponding calibration curves (right). Left: blue = antibody, green = analyte (open) and analyte derivative (closed), red = label. Right: c = concentration, P = unknown sample, S = signal emitted by the antibody–analyte tracer complex (a,b), antibody tracer–analyte derivative complex (c,d) and from the sandwich complex (e,f). This and other schematic representations of an antibody do not take into account the existence of several binding sites (in the case of the IgG antibody: two).

Group II: Competitive immunoassays with antibody tracer:

As in Group I this is also a competitive process. The only difference is that of the two detection reagents (antibody, analyte derivative) it is not the analyte derivative that carries the label but the antibody (antibody tracer also called tracer antibody). As can be seen from Figure 3c, the concentration of the analyte to be determined correlates with the concentration of the antibody tracer-analyte derivative complex after adjusting the equilibrium (Fig. 3d). For the separation of the two labeled complexes which is also necessary in this case see Section 6.1.3.

Group III: Sandwich assays:

Instead of the analyte derivative in competitive assays, in this case a second antibody is the detection reagent. The label is situated on one of the two antibodies. Both antibodies bind to the analyte at different sites (epitopes) and thus form a sandwich complex. An excess of the two antibodies is employed in order to shift the equilibrium in favor of the sandwich complex (Fig. 3e). The favorable equilibrium position in this type of assay due to the excess of reagent leads to a considerably higher sensitivity of detection. Whilst, for example, a competitive assay for the determination of the thyroid hormone thyrotropin is capable of detecting an analyte concentration of about 1–2 fmol mL^{-1}, the lower detection limit in the case of a comparable sandwich assay is about 0.1–0.2 fmol mL^{-1}. The sandwich strategy achieved its breakthrough when it became possible to obtain pure uniform antibodies in virtually any quantity. (Monoclonal antibodies, Nobel Prize, 1984 for Köhler and Milstein).[102, 103]

About the only disadvantage is the limited applicability of this type of assay: small analytes (M < approx. 5 kD) are excluded, since two antibodies cannot bind simultaneously for steric reasons. In the case where the analyte is an antibody (cf. listing in Section 6.1.1; indication: infectious diseases), the sandwich assay can be applied in a slightly-modified form: an antigen plays the part of one of the two detection antibodies, thus, for example, a virus particle, against which the analyte antibody is directed. In this case, the resulting sandwich complex consists of antigen, analyte antibody, and detection antibody; the antigen corresponding to its complementary structure binds to the recognition site of the analyte antibody and is, thus, responsible for the specificity of the detection. For the detection antibody it suffices if this is directed against the structure element of the analyte antibody, although this is standard for all

antibodies of the same animal (in this case human) species (Fc portion). The latter presupposes, of course, that the antibodies present in the patient's blood, which are directed against completely different antigens, do not "capture" a significant fraction of the detection anti body. In practice such complications can be easily avoided by the choice of a corresponding high concentration of the detection antibody or by a so-called two-step performance of the assay. The separation of the sandwich complex and excess tracer, also necessary in this case prior to the measurement, is dealt with in Section 6.1.3.

The individual variations within these groups (cold and hot preincubation, one-step and two-step performance, double antibody method) as well as immunoassays which work without a tracer[1,8a] cannot be dealt with here.

Definitions of Terms:

In scarcely any other area is there such a confusion of terms and abbreviations as in the field of immunoassays. The most well-known expression is the "radioimmunoasssay" (RIA). Unfortunately it is often used for two different circumstances. First, it stands for competitive immunoassays with a radioactive analyte tracer, that is, for the assay type described in Group I, and second it is often employed as the generic term for all immunoassays with a radioactive label. The same can also be said for the enzyme immunoassay (EIA), fluorescence immunoassay (FIA), and luminescence or chemiluminescence immunoassay (LIA and CIA, respectively) which are distinguished from RIA only by the type of label used. A similar ambiguity exists with the acronym ELISA (*enzyme-linked immunosorbent assay*). This term is reserved by some authors for the excess reagent assay (Group III) with the enzyme label and by others is employed quite generally for all immunoassays with enzyme label.

Just as XIA (RIA, EIA, FIA, LIA, CIA) designates specifically assays of Group I, Group II does not have a generally accepted abbreviation. However, in this case one often comes across the expression SPALT (*solid phase antigen luminescence technique*). This describes an assay of Group II with luminogenic label, for which a particular but frequently used technique for separating excess antibody tracer is employed (see Section 6.1.3).

A synonym for sandwich assay (Group III) is the expression 2-site IXMA; this stands for "immuno-x. . . .metric assay" (for example: IRMA: immunoradiometric assay; ILMA: immunoluminometric assay). The expression immunometric means that, in contrast to the competitive assays, one is dealing with an assay with excess reagent. Unfortunately the designation is also not uniform in this case. Hence, Group II, despite its competitive nature, is still designated with the expression "1-site IXMA". Strictly speaking the 1-site IXMA is, however, a very rare type of assay, which uses the same detection reagents as Group II (analyte derivative and antibody tracer), however, uses the antibody tracer in excess, and does not measure the complex formed from the analyte derivative and antibody tracer, but the complex formed from the analyte and antibody tracer.

6.1.3. *Separation Methods*

As already mentioned the selective measurement of labeled immune complexes necessitate a prior separation of the unbound analyte tracer (in the case of Group I), of antibody tracer not bound to the analyte derivative (Group II), or of unbound antibody tracer (Group III).

The first separation methods involved really difficult purification steps, for example chromatography or electrophoresis. Considerably more manageable, but today regarded as being antiquated, are the methods in which the immune complexes are precipitated by addition of salts or organic solvents, or the unbound analyte tracer is adsorbed on addition of activated charcoal or ion exchange resin. The requisite centrifugation step renders these methods as being no longer able to compete today.

Modern methods usually employ a solid phase. In the simplest case this is a small tube made of synthetic material, whose inner wall is coated with one of the detection reagents (coated tubes). At the same time these tubes serve as the reaction vessel for the immunological detection reaction. The separation immediately prior to measurement is reduced to just merely decanting off or removal of the reaction solution by suction (Fig. 4).

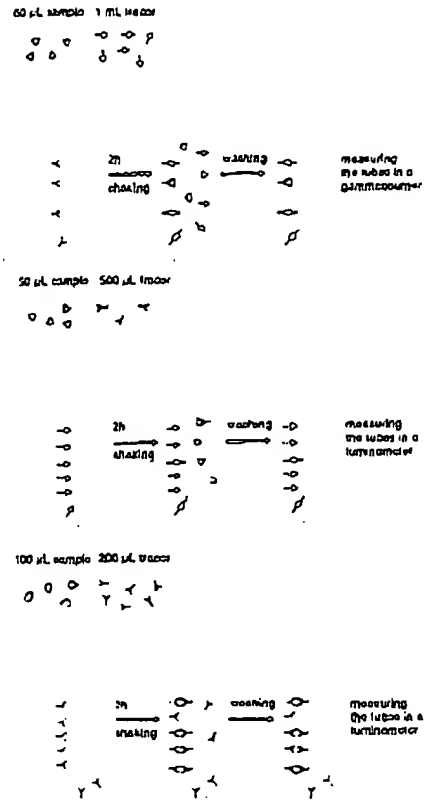


Fig. 4. Examples of commercial immunoassays performed with a coated tube: RIA-gnost T3 (top), a RIA and BeriLux T3 (middle), a SPALT assay for determination of triiodothyronine (T3) and BeriLux TSH (bottom), a 2-site ILMA for the determination of thyroid stimulating hormone (TSH) in serum.

The coating of the solid phase proceeds in the most simple case by direct adsorption of the detection reagent; the solid phase is kept in contact with a solution of the detection reagent for several hours. Under suitable conditions the adsorptive binding is so strong that the immobilized reagent cannot be dissolved by washing the solid phase. Binding can also result through an anchor protein, which adsorbs particularly well onto

the solid phase to which the detection reagent is covalently bonded by bifunctional reagents.[24] Often an antibody, which recognizes a structural element that is common to all antibodies of another animal species, is attached to the solid phase. For instance, antibodies which are directed against the Fc portion of mouse antibodies can be produced in rabbit. In this way antibodies can be anchored onto the solid phase which are less suitable for a direct adsorption. More of these universal solid phases are based on the high affinity binding between biotin and avidin (strept-)avidin[34] as well as between fluorescein and anti-fluorescein antibodies[184] (Fig. 5).

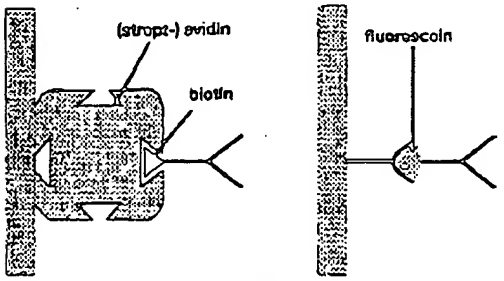


Fig. 5. The binding of a detection reagent (in this case an antibody) to a solid support can be achieved by means of the (strept-)avidin/biotin system (left): The biotinylated antibody is supported by the (strept-)avidin adsorbed onto the solid support. A similar anchoring method is based on the strong bonding between fluorescein groups and antibodies directed against fluorescein (right).

Instead of coated tubes other coated solid phases can also be employed (synthetic spheres, magnetic particles, and membranes). These can also (cf. Fig. 4) participate directly in the immune reaction or merely have a separation function. In the latter case the immune reaction occurs in a homogeneous liquid phase (which has certain advantages with regards to the rate of reaction), and the separation function of the solid phase is only "switched on" once the reaction is completed (Fig. 6).

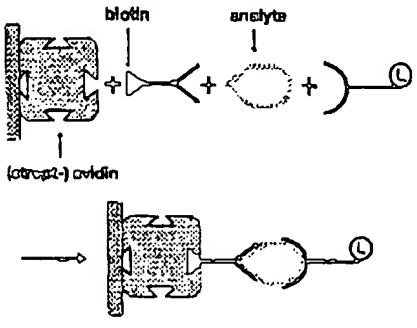


Fig. 6. After completion of the reaction between antibody labeled with either FITC or biotin, analyte, and analyte tracer in the liquid phase, the solid support coated with (strept-)avidin or anti-fluorescein antibodies, respectively, is added, and the sandwich complex is attached to the solid support.

With the OPUS system[185] separation is accomplished without further operation steps. The whole immune reaction takes place in a test module which is about the size of two sugar cubes. An essential component is a transparent polyester film, on the surface of which are three agarose layers. Complexes of the antibody and fluorescent-labeled analyte derivative are embedded in the lower layer (1 μm thick). The serum sample is applied to the top layer (10 μm thick). The analyte molecules contained in the sample diffuse into the lower layer, where they—depending on their concentration—displace more or less tracer molecules from the antibody binding sites. Additional reagents such as aqueous solutions are not necessary here. Whereas antibodies are not able to leave the lower layer because of their size, the unbound tracer molecules are free to diffuse into the two upper layers. The antibody–tracer complexes which remain in the lowest layer are quantified by fluorescence detection. The light source and detector are situated below the test module. The middle agarose layer (10 μm thick) acts as an optical filter due to its iron oxide content and prevents measurement of released tracer molecules which have diffused from the lower layer.

In the purely homogeneous assays there is no separation step because they are based on a changing signal in the formation of the immune complex. The first assay of this type was presented by Rubenstein and Ullman in 1971.[186a] This EMIT method (enzyme-modulated immunoassay technology) involves EIA; enzymatic activity of the enzyme label is inhibited by the binding of antibodies (Fig. 7). Another example of a homogeneous as-

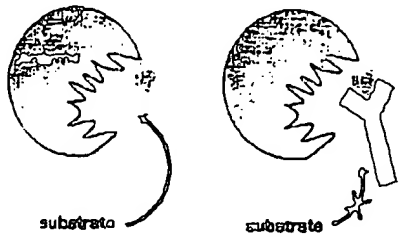


Fig. 7. In the EMIT method an antibody bound to the analyte tracer (analyte derivative labeled with the enzyme) prevents the catalytic conversion of the substrate (e.g. a chromogen) by the enzyme, for example, by steric hindrance at the active site.

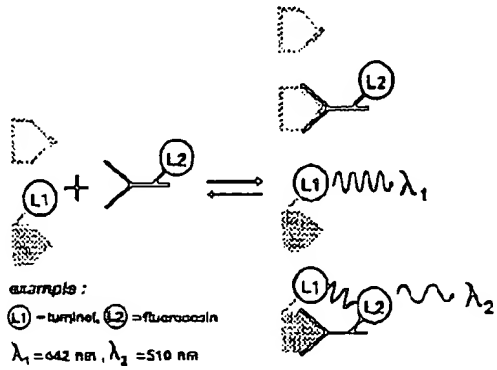


Fig. 8. Example of a homogeneous EIA: The energy transfer across the small distance between the two labels L1 and L2 results in an electronic excitation of L2. The intensity of the light emitted from L2 therefore correlates to the analyte concentration.

say which employs chemiluminescence energy transfer is shown in Figure 8. Disadvantages of these, at first glance, particularly elegant homogeneous assays are lower sensitivity of detection and a more pronounced susceptibility to interference.

6.2. Gene Probes

Whereas analytes in immunological determination methods are after all products of genetic information, nucleic acid sequences are the information itself. The qualitative and quantitative determination at this "primary level" is increasingly gaining significance.

The most important application at present is in the detection of pathogenic organisms (bacteria, viruses).[187] A particular advantage is that not only active viral infections, but also latent ones, are ascertainable by detection of the nucleic acid sequences. Thus, for example, infections with the AIDS virus can be detected already in the incubation phase of seronegative patients. In addition, the control of blood supplies for HIV, HTLV-I, Hepatitis B, etc. is much safer with the detection of the corresponding nucleic acid sequences than with an immunological test.

A series of hereditary diseases, such as diabetes mellitus, Lesch–Nyhan syndrome, phenylketonuria, and sickle cell anemia, can be detected reliably by tracing the mutated genes,[188] likewise the activation of different oncogenes which are involved in the formation of tumors.[189] In forensic medicine the detection of nucleic acid sequences is employed for solving cases involving sexual crimes or in tests for paternity suits.[190]

In contrast to immunoassays no antibodies are employed as direct detection reagents, but relatively short, mostly synthetic nucleic acid sequences (so-called gene probes) which are complementary to part of the analyte (so-called target sequence) (Fig. 9). These hybridize with the target sequence, that is, they

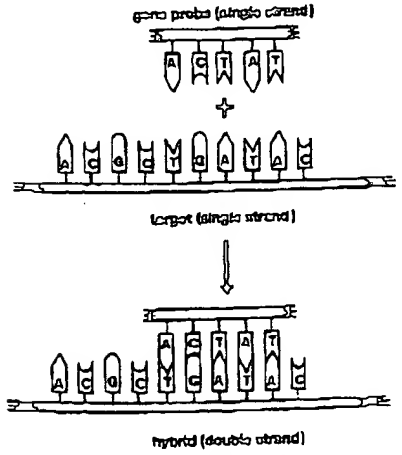


Fig. 9. The affinity of two nucleic acid strands arises from complementary base pairs: adenine (A) bonds to thymine (T) or uracil (U), and cytosine (C) to guanine (G). The gene probes employed are usually chains of 15–30 nucleotides.

bind with it to form a double strand which is held together by hydrogen bonding.

In the Southern–Blot method,[191] named after its founder, the DNA, on which presumably the sequence to be determined lies, is initially cut into defined fragments by restriction enzymes. These are separated by electrophoresis and the bands on the electrophoresis gel are transferred to a suitable carrier (e.g. nitrocellulose), whilst maintaining the relative positions of the bands. The individual DNA fragments (double-stranded) are denatured (that is, they are split into single strands) by heating, in order to make them accessible to a hybridization with the labeled gene probe. One of the advantages of this method is that different probes can be employed simultaneously to trace several nucleic acid sequences.

The visualization of the fragments hybridized with the probes can be accomplished, for example, by applying a photographic film. The exposure times are greatly dependent on the label employed. For the ^{32}P isotope used almost exlusively earlier, it was not unusual to have to wait for several weeks. By employing more modern luminogenic labels the exposure times can be drastically reduced. Methods that use fluorescent labels dispense with need for photographic film and, moreover, there is the advantage that different colored light signals can be received by employing different labels simultaneously. This can make the distinction of DNA fragments which exhibit similar electrophoresis profiles considerably easier.

In analogy to immunoassays there are also corresponding gene probe tests. The pendant of a 2-site IXMA[192] is shown in Figure 10. An analogue of the competitive immunoassay is the

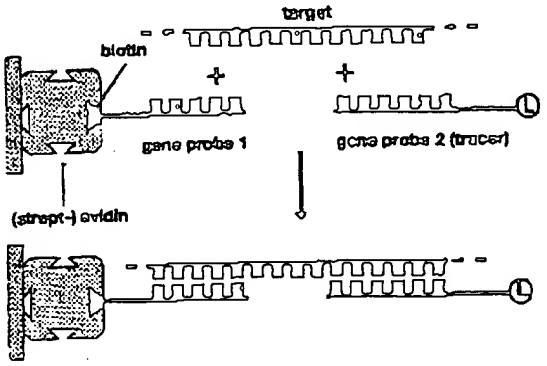


Fig. 10. The counterpart corresponding to the 2-site IXMA on gene probe site.

strand displacement assay[193] (Fig. 11). The only difference, in principle, to the XIA presented in Section 6.1.2. (Group I) is that the labeled gene probe (corresponds to the analyte tracer in XIA) and the target sequence (analyte) do not compete at the same time for the probe bound to the solid phase (corresponds to the antibody in XIA), but that the tracer gains a lead in time (principle of hot incubation; not an unusual experimental variable in XIA).

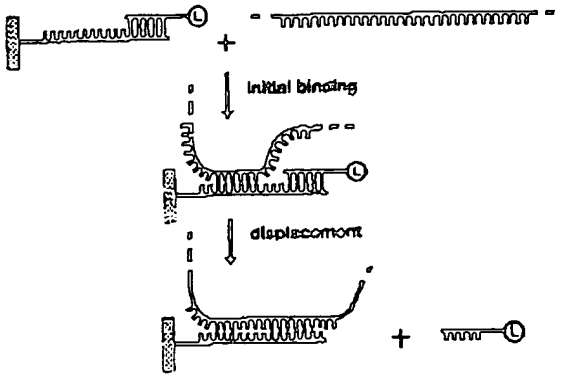


Fig. 11. Principle of the strand displacement assay.

The transfer of techniques from the field of immunoassays has also led to homogeneous gene probe test. In kissing probes (Fig. 12), two labeled probes bind to the target so closely to each other that an interaction is effected between the labels. Thus, only the changes in the signal occurring in this way can be viewed as highly specific.

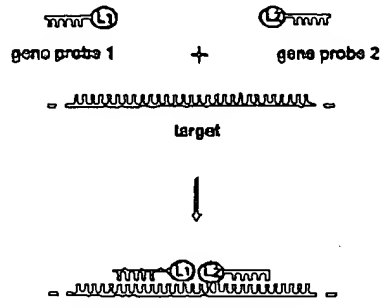


Fig. 12. Principle of the "kissing probes".

The acridinium label is able to intercalate double-stranded DNA and therefore is protected against attack from nucleophilic reagents. In this way it is possible to distinguish between single-stranded and double-stranded bound labels. This has been of use in the "hybridization protection assay".[194]

A revolutionary step for enhancing the sensitivity of gene probe tests was achieved by the PCR methods (polymerase chain reaction).[195] K. B. Mullis received the Nobel prize for the development of this method in 1993. The basic idea is original and at the same time simple. Whereas almost all attempts to improve the lower detection limit were directed at increasing the signal intensity and reducing the background signal, that is, to have the tracer in sight, with the PCR method, the target sequence is selectively replicated(!) and moreover quite simply. First, the target sequence present as a DNA double strand is cleaved into two single strands by denaturing with heat. The two single strands were then hybridized with complementary oligonucleotides and, these were subsequently enzymatically

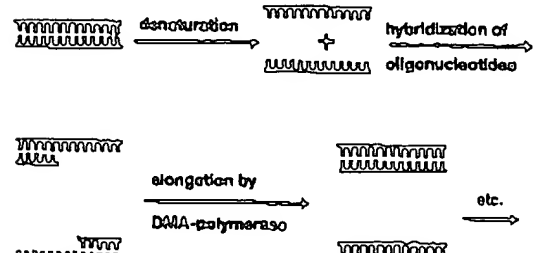


Fig. 13. PCR method.

elongated at the 3'-end with deoxynucleotide triphosphates. This process is repeated several times (Fig. 13) and after 20 cycles the target sequence is amplified by 100 000-fold.

7. Outlook

Even if a γ source which radiates continually for months to emit a signal often only required for a few seconds is everything but modern, there is much to be said, not only on ecological grounds, for the replacement of radioactive labels. With the labeling alternatives available today radioactive labels have, as far as detection sensitivity is concerned, been partly superseded. Why is it then that RIA did not die out long ago? A considerable advantage of RIA is tied up with one of its greatest disadvantages—as seen from an ecological standpoint—the emission of energy-rich radiation. In the thirty years of experience in the field of RIA this method has proved to be extraordinarily "robust". Thus, for example, in the case of the radioactive label the tracer is not influenced by its direct surroundings ("matrix effects") except for in the rarest of cases. Furthermore, the signal is not affected either in terms of its absolute value or in terms of its constancy by most external factors. In addition, the labeling of small analyte derivatives by an isotope inevitably results in fewer changes in properties compared to the introduction of a sterically demanding label. Thus, replacement of radioactive methods will understandably not occur overnight, but is more of a gradual process which is sustained by a steady increase in experience in dealing with nonradioactive labels and the synthesis of more effective labels. Since the most efficient luminescent labels and luminogenic enzyme substrates have only been available for a few years, one can expect an acceleration of this hitherto slow replacement of radioisotopes in the years to come.

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